The role of adipose tissue in endocrine and metabolic diseases:

studies in men and mice

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Chapter 1

General introduction and outline of the thesis

Obesity and related metabolic disorders

Obesity is characterized by an excessive accumulation of fat mass. According to the World Health Organization the prevalence of obesity has reached epidemic proportions worldwide nowadays, with more than 1 billion adults being overweight (1). The prevalence of overweight and obesity is assessed by using the body mass index (BMI) or waist to hip ratio (WHR), defined as the weight in kilograms divided by the square of the height in meters (kg/m^2) or maximum waist circumference divided by the maximum hip circumference, respectively.
Important causes of obesity are sedentary lifestyle habits and easy accessibility to high energy-dense foods. When energy intake exceeds energy expenditure, the excess amounts of energy will be stored in the adipose tissue. Oppositely, in case of energy demand, adipose tissue is able to liberate fatty acids that serve as energy source by inducing lipolysis (2). Besides environmental determinants, genetic factors play an important role in the predisposition to obesity. It has been shown that variations in genes like leptin, peroxisome proliferator- activated receptor (PPAR)-γ and fat mass and obesity associated gene are more associated with the susceptibility to gain weight (3-6). During obesity, adipose tissue is considered as one of the key players in the development of a disturbed glucose- and lipid homeostasis (7). In this chapter the physiological and pathophysiological aspects of adipose tissue will be described in relation to obesity and associated metabolic disorders.

Adipose tissue composition and distribution

Adipose tissue composition

Adipose tissue is composed of a heterogeneous cell population which can roughly be divided into two groups: the mature adipocytes and the stromal vascular fraction (SVF) (8). The primary functions of mature adipocytes are to regulate lipogenesis and lipolysis (9;10), and to secrete many adipose tissue-derived hormones named adipokines (11;12). During obesity, adipose tissue mass expansion occurs by both an increase in adipocyte cell size (hypertrophy) and by inducing the number of adipocytes (hyperplasia) (13). Metabolic abnormalities like adverse changes in glucose- and lipid metabolism which are associated with obesity, have been found to be related to adipocyte hypertrophy rather than to adipocyte hyperplasia (14;15). Adipocyte hypertrophy is associated with cellular dysfunction, leading to impaired glucose metabolism, insulin resistance and eventually type 2 diabetes mellitus (16;17).
The SVF consists of a variety of cell types including pre-adipocytes, fibroblasts, endothelial cells, yet also accommodates all sorts of immune cells including: monocytes, macrophages and lymphocytes (18;19). Pre-adipocytes have the potential to differentiate into new mature adipocytes (20). Impaired differentiation of pre-adipocytes is

accompanied by a diminished lipogenesis of newly formed adipocytes that may result in hypertrophy of existing mature adipocytes (21). Furthermore, obesity is associated with a change in cellular composition of the SVF in adipose tissue (22). This is characterized by an increased influx of different immune cell populations secreting many pro-inflammatory mediators, which contribute to an adverse metabolic profile that leads to insulin resistance in the adipose tissue. This process will be described in more detail later in this chapter.

Adipose tissue distribution

Recently, magnetic resonance imaging scanning revealed that subjects who have a normal body weight can suffer from enormous fat accumulation around the organs (23;24). These individuals are described as TOFIs (thin on the outside, fat on the inside). Adipose tissue can be stored either subcutaneously (in the buttocks, thighs and abdomen) or viscerally (around the omentum, intestines and perirenal areas). It has been shown that especially an increase in the visceral adipose tissue (VAT) compartment enhanced the risk of type 2 diabetes mellitus and cardiovascular disease (CVD) (25-27), whereas an increase in subcutaneous adipose tissue (SAT) depot exerts no risk or even has a protective effect against obesity-induced metabolic disturbances, presumably by preventing ectopic accumulation of lipids (28-30). Therefore, not solely the amount of fat mass is important, but especially the distribution of fat throughout the body determines its harmful effects. Two major assumptions have been made that may explain the adverse metabolic consequences of VAT. The first is based on the anatomical location of VAT and its capacity to deliver free fatty acids (FFAs) and various adipokines into the portal circulation were they can directly affect liver metabolism (31). The second assumption considers the intrinsic cellular properties in VAT, regarding decreased adipocyte differentiation capacity, and the increased release of unfavorable adipokines and pro-inflammatory mediators compared to SAT (32-35).

Adipose tissue as an active endocrine organ

As already briefly mentioned, adipose tissue is an active endocrine organ secreting many adipokines that are able to modulate energy homeostasis (energy intake and expenditure), glucose- and lipid metabolism. Two of the most well studied adipokines are adiponectin and leptin. Adiponectin is exclusively produced by adipocytes and high serum levels of this adipokine are associated with increased insulin sensitivity (36). Adiponectin circulates in the plasma in various multimeric forms that include the low molecular weight (LMW), middle molecular weight (MMW) and the high molecular weight (HMW) isoforms (37). It has been shown that especially the HMW form of adiponectin acts as an insulin sensitizer in an autocrine fashion, but also promotes

fatty acid oxidation and glucose uptake of the muscle and liver (38;39). Furthermore, adiponectin exhibits anti-inflammatory and anti-atherogenic properties, which is illustrated by the inverse relationship between circulating concentrations of this protein and the incidence of CVD (40). Leptin is also mainly produced by adipose tissue and plays an important role in controlling food intake and energy expenditure via leptin receptors in the neural regions of the hypothalamus (41). Furthermore, leptin influences immune functions by regulating T-lymphocyte response (42).
Excessive fat accumulation is associated with a disturbed adipokine secretion pattern. As previously discussed, the increased number of large adipocytes may be responsible for this altered production (16;17). The expression level of adiponectin is significantly reduced in large adipocytes, whereas leptin levels, and the more harmful adipokines resistin and visfatin, are elevated in hypertrophic cells (43-45). Alterations in adipokine production that promote adverse metabolic effects are more pronounced in expanding VAT compared to SAT (46). All together this implicates that obesity, associated with an increase in VAT, results in an adverse endocrine secretion profile that could lead to metabolic disturbances. Interestingly, most of the adipokines produced by the expanded adipose tissue are pro-inflammatory mediators, suggesting that adipose tissue also acts as an immunologically active organ (47).

Adipose tissue as an inflammatory organ

It is now generally accepted that obesity is accompanied by the development of a chronic state of low-grade inflammation. Both animal and human studies revealed that obesity is associated with elevated circulating levels of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, high sensitive C-reactive protein (hsCRP) and interleukin (IL)-6, which have been demonstrated to induce insulin resistance (48;49). In fact, weight loss in obese subjects results in a reduction of systemic inflammatory cytokine levels and an improvement in insulin sensitivity (50). Together, these observations support the close link between inflammation and insulin resistance (51-53) and suggest that expanding adipose tissue strongly contributes to the development of obesity-induced inflammation. Indeed, adipose tissue has been recognized as an important instigator of obesity-induced inflammation (54;55). The inflammatory properties of adipose tissue have been discovered since the revolutionary study performed by Spiegelman et al. in 1993, showing that adipose tissue from animals with diabetes and obesity is able to secrete TNF-α (56). The concept of adipose tissue as an important source of pro-inflammatory mediators rapidly expanded with the identification of IL-6, IL-8, plasminogen activator inhibitor (PAI)-1 and monocyte chemo-attractant protein-1 (MCP-1) as drivers of metabolic adipocyte dysfunction (57-59). Concerning the different adipose tissue depots, VAT seems to contribute to a larger extend to these elevated cytokine levels than SAT (57;60). Currently, less is

known about triggers that instigate the inflammatory response in adipose tissue and the possible sensors that detect these triggers, although some progress has been made by the identification of elevated levels of glucose and FFAs as inducers of several pro-inflammatory adipokines (61-63).

IL-1 family of cytokines in obesity-induced inflammation

The class of IL-1 family cytokines has been shown to play an important role in obesity-induced inflammation that promotes the development of insulin resistance and type 2 diabetes mellitus. Plasma levels of the prominent IL-1 family members IL-1β and IL-18 are increased during obesity and associated with insulin resistance (64-67). IL-1β is a pro-inflammatory cytokine having insulin desensitizing effects in liver and adipose tissue and causes pancreatic beta-cell failure (66;68;69). Oppositely, IL-18 has been shown to improve insulin sensitivity and control food intake (70;71). The adipose tissue is also able to produce IL-1β and IL-18 (64;72), yet the underlying mechanism or triggers that control the release of these cytokines from adipose tissue remains unknown. However, in several cell types that are known to produce high amounts of cytokines including monocytes, macrophages and dendritic cells, inactive pro-IL-1β and pro-IL-18 are cleaved into its mature active form by a cysteine protease called caspase-1 (73). This enzyme requires activation by an intracellular multi-protein complex termed the inflammasome. The most fully characterized inflammasome complex consists of caspase-1, the cytoplasmic pattern recognition receptor named nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) protein 3 (NLRP3), and the adapter protein apoptosis-associated specklike protein (ASC) (74). Lately, inflammasome-generated IL-1β secretion has also been ascribed to pancreatic beta-cells that mediates the development of insulin resistance, with a crucial role for the protein thioredoxin interacting protein (TXNIP) in controlling the release of IL-1β (75). This protein, which acts as an inhibitor of the reactive oxygen species (ROS) scavenging protein thioredoxin, is expressed in different cell types, including adipocytes, and is elevated in subjects with type 2 diabetes mellitus (76;77). The authors have shown that upon induction of ROS, TXNIP is released from thioredoxin and can interact with NLRP3 to activate caspase-1, which leads to the release of IL-1β in mouse pancreatic beta-cells (75).

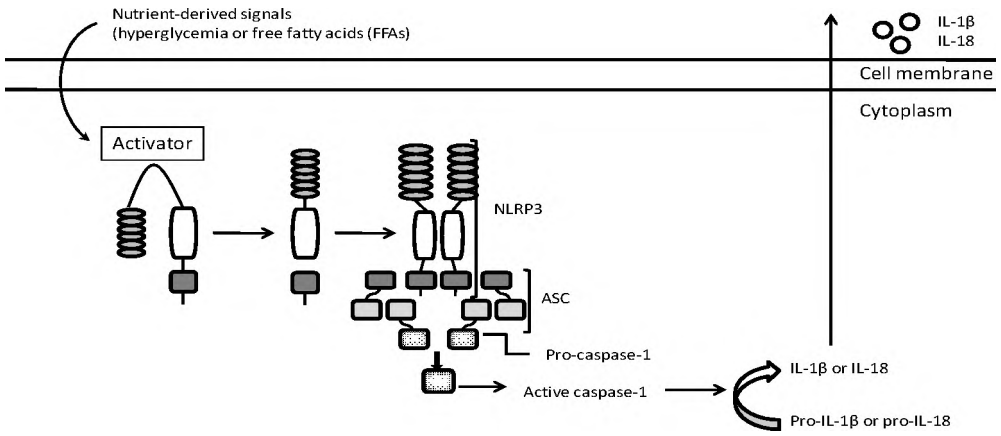

Figure 1 **A model of NLRP3 inflammasome activation by nutrient-derived signals.** The NLRP3 inflammasome is activated by nutrient-derived triggers including high levels of glucose and free fatty acids. These signals directly or indirectly activate NLRP3. NLRP3 oligomerizes and provokes the recruitment of ASC. This signals the assembly of pro-caspase-1, which is cleaved by a process called auto-cleavage into active caspase-1. Active caspase-1 is able to process the pro-forms of IL-1β and IL-18 into their active forms, which are secreted from the cell.

Cellular sources of pro-inflammatory mediators in adipose tissue

Like other inflammatory responses, obesity-induced inflammation leads to infiltration of a variety of immune cells into the inflamed tissue. In the last ten years, it has been clarified that the infiltration of macrophages into adipose tissue plays an important role in the onset of obesity-induced inflammation. Increased numbers of adipose tissue macrophages (ATMs) are robustly associated with elevated levels of pro-inflammatory mediators including TNF-α, MCP-1, IL-6 and IL-8 and the development of insulin resistance (22;78). In line with these observations, a reduction in ATMs results in a diminished inflammatory response together with an improvement in insulin sensitivity (79;80). However, recent evidence suggests that ATMs are not the only immune cells responsible for the inflammatory response during obesity. Neutrophils represent one of the most prominent components of the innate immune system, displaying strong phagocytotic and antimicrobial activity. It has been demonstrated that the number of neutrophils and its activation state is higher in severely obese subjects (81). Besides immune cells belonging to the innate immune system, involvement of the adaptive immunity has also been linked to adipose tissue inflammation. Recently, it has been demonstrated that T-lymphocytes ($CD3^+$ T-cells) accumulate in obese adipose tissue due to large numbers of $CD8^+$ T-cells (cytotoxic T-lymphocytes) and the pro-inflammatory T helper (Th)-1 cell subset of $CD4^+$ T-cells (82-84). Furthermore, infiltration of cytotoxic T-lymphocytes into the adipose tissue has found to precede the accumulation of macrophages, suggesting an essential role of T-cells

in the initiation and maintenance of adipose tissue inflammation (85;86). In contrast, numbers of the anti-inflammatory Th-2 cells and regulatory T (Treg)-cells are found to be lower in adipose tissue of obese animals (87;88). Influx of immune cells into adipose tissue also appears to be depot specific since macrophage and T-cell infiltration is more pronounced in VAT as compared to SAT (85;89;90). Therefore, the influx of immune cells in this particular fat depot might also contribute to its pro-inflammatory character.

Besides the immune system, pre-adipocytes and adipocytes themselves contribute to the obesity-induced inflammation. It has been shown that hypertrophic adipocytes have a disturbed secretion pattern of pro- and anti-inflammatory cytokines leading towards a more pro-inflammatory profile (44). Moreover, impaired differentiation from pre-adipocytes into mature adipocytes has been associated with a more pro-inflammatory profile and enhanced release of chemokines, which are critical for the recruitment of immune cells into the adipose tissue (91). In this way, the enhanced inflammatory response associated with dysfunctional adipose tissue could link obesity to metabolic disorders characterized by insulin resistance.

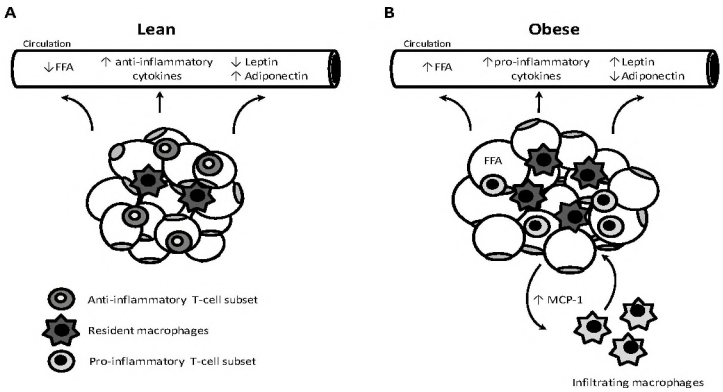

Figure 2 **Alterations in adipose tissue morphology contributes to obesity-induced inflammation.** (A) In lean subjects, adipose tissue is characterized by small insulin sensitive adipocytes and the presence of anti-inflammtory Treg cells and Th2 cells. This is accompanied with elevated levels of adiponectin and anti-inflammatory cytokines including IL-10 and IL-1 receptor antagonist that suppresses inflammation and improves insulin sensitivity (B) In obese subjects, adipose tissue is characterized by adipocyte hypertrophy with an increased release of FFAs and pro-inflammatory adipokines including leptin, IL-6, IL-8, TNF-α and MCP-1. These mediators are released into the circulation and mediates the infiltration of macrophages and pro-inflammatory cytotoxic T-cells and Th1 cells in the adipose tissue. This further amplifies the immune response in the adipose tissue eventually resulting in local and systemic insulin resistance.

Obesity and related metabolic disorders

Adipose tissue inflammation predisposes to the development of insulin resistance and several other metabolic abnormalities including dyslipidemia and hypertension (43). Insulin resistance is defined as a diminished ability of a cell to respond to insulin that will subsequently lead to a reduced uptake of glucose. To compensate, the pancreas starts to produce increasing amounts of insulin. Eventually, insulin resistance may develop into type 2 diabetes mellitus when insulin-producing pancreatic beta-cells can no longer compensate for the decrease in tissue insulin sensitivity. In addition, insulin resistance is closely linked to the pathogenesis of chronic diseases such as metabolic syndrome, familial combined hyperlipidemia (FCH) and cardiovascular diseases (CVD) (92-94).

As discussed above, pro-inflammatory cytokines are key players that link obesity-induced inflammation to insulin resistance and recent studies provided more insight into the underlying mechanisms. Elevated cytokine levels could inhibit insulin signaling pathways in metabolically active organs, such as liver, pancreas and muscle, by blocking Insulin Receptor Substrate-1 phosphorylation (95). Furthermore, inflammatory mediators might lead to the down-regulation of certain genes involved in the insulin signaling cascade and to the augmentation of lipolysis (96). This results into increased levels of circulating FFAs that may promote ectopic fat deposition that further amplifies systemic insulin resistance. The central role for the pro-inflammatory cytokine IL-1β in the pathophysiology of insulin resistance has been supported by results from a clinical trial in which patients with type 2 diabetes mellitus receiving treatment with IL-1 receptor antagonist display an improvement of glycemic control (97). Another class of insulin sensitizing pharmaceutical compounds known as thiazolidinediones (TZDs) have been described, which are agonists of PPAR-γ. TZDs induce glucose uptake transporter (GLUT)-4 expression via PPAR-γ activation and restore adipocyte lipogenesis and differentiation (98;99). Furthermore, TZDs possess anti-inflammatory activities by increasing the PPAR-γ target adiponectin and reducing inflammatory gene expression (100). These findings indicate that anti-inflammatory therapies are valuable to reduce obesity-associated inflammation.

Outline of the thesis

The central aim of this thesis is to further investigate the role of adipose tissue adipokines and inflammation in the etiology of metabolic disorders associated with insulin resistance in both mice and man. In the first two chapters we studied adipose tissue dysfunction in two different patient populations. First, we described the pathogenic role of adipose tissue in patients suffering from FCH (**chapter 2**). To analyze adipocyte dysfunction, we studied the adipocyte-specific secretion of the metabolically favorable HMW isoform of adiponectin in the circulation of FCH subjects and healthy controls. In a second study, we investigated whether the insulin sensitive effects of the TZD pioglitazone were related to a more beneficial adipocyte morphology and metabolic profile of SAT in patients with congenital adrenal hyperplasia (CAH), who are characterized by insulin resistance (**chapter 3**).
The role of two prominent members of the IL-1 cytokine family: IL-1β and IL-18 and their intracellular processor, the inflammasome-mediated caspase-1 were studied in chapter four to seven. We determined the role of these components in adipose tissue inflammation and controlling insulin sensitivity. Since IL-1β and IL-18 have contradictory effects on insulin sensitivity, we first studied the contribution of IL-18 to the development of obesity-induced insulin resistance by feeding both wild-type and IL-18-/- animals a high fat-diet (**chapter 4**). Because IL-1β and IL-18 affect metabolic homeostasis and due to the essential role of caspase-1 in activating these two cytokines, we next studied the possible role of caspase-1 as a potential key player in regulating adipose tissue inflammation and insulin sensitivity (**chapter 5**). In addition, we focused on hyperglycemia as a potential trigger of caspase-1 in human adipose tissue that may contribute to chronic inflammation and insulin resistance via IL-1β (**chapter 6**). Furthermore, we investigated the involvement TXNIP in hyperglycemia-induced IL-β secretion by human adipocytes. Finally, we determined whether the inflammasome components NLRP3, ASC and caspase-1 are more active in human VAT compared to SAT, which may partly explain the previously reported differences in the pro-inflammatory character between both adipose tissue depots (**chapter 7**).

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Chapter 2

Adiponectin Multimer Distribution in Patients with Familial Combined Hyperlipidemia

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Abstract

Introduction: Adiponectin is secreted from adipocytes in different multimers, of which the high molecular weight (HMW) form is supposed to mediate favourable metabolic and anti-atherogenic effects.

Materials and Method: We determined adiponectin multimers in 29 female and 22 male patients with familial combined hyperlipidemia (FCH) and 51 age-, gender- and BMI-matched controls in relation to cardiovascular disease (CVD).

Results: We observed a clear sexual dimorphism of total adiponectin and its multimers. Female, but not male, FCH patients had significant lower total adiponectin and both HMW and low molecular weight (LMW) adiponectin than controls.

Conclusions: The adiponectin sensitivity index (ASI), reflected by HMW/total adiponectin, and the LMW/HMW adiponectin ratio did not differ significantly between FCH females and control females. However, FCH females with CVD exhibited significantly lower ASI (34.2 ± 10.1% vs 46.0 ± 7.1%) and higher LMW/HMW ratio (1.5 ± 0.8 vs 0.7 ± 0.3) compared to FCH females without CVD, reflecting a more atherogenic adiponectin multimer distribution.

Introduction

Adipose tissue is an active endocrine organ secreting many biologically active substances (adipokines) (1). Adiponectin is an adipokine which is adipocyte-specific and is abundantly present in the circulation (2). After post-translational modifications, adiponectin is secreted into the circulation in three different multimers: a low molecular weight (LMW) form, a middle molecular weight (MMW) form and a high molecular weight (HMW) form (3;4). Previous studies reported that circulating levels of total adiponectin are decreased in disorders associated with obesity, dyslipidemia, insulin resistance and inflammation (5-9). In the last years, many studies have also described an association of adiponectin deficiency with increased incidence of coronary heart disease (10;11). This is in agreement with the observation that high levels of adiponectin are associated with a reduced risk of atherosclerotic plaque formation (12). Furthermore, experimental data suggest that adiponectin is involved in prevention of foam cell formation, down regulation of adhesion molecules, inhibition of endothelial dysfunction, and smooth muscle cell proliferation and migration (13-16). Therefore, adiponectin is supposed to be protective against cardiovascular diseases (CVD).
Recently, we reported that total plasma adiponectin is decreased in patients with familial combined hyperlipidemia (FCH), even after adjustment for body adiposity and degree of insulin resistance (17). FCH is the most common heritable, multifactorial lipid disorder with a prevalence of 1-5% in the general population. The disturbed lipid profile seen in patients with FCH is characterized by elevated levels of total cholesterol (TC), triglycerides (1) and apolipoprotein B (apoB). Other characteristic features are increased levels of low-density lipoprotein cholesterol (LDLc), decreased levels of high-density lipoprotein cholesterol (HDLc) and the presence of small dense LDL (sdLDL) (18-20). In addition, FCH patients are often obese and insulin resistant (21). So, FCH patients are exposed to several cardiovascular risk factors which contribute to the 2-5 fold increased risk to develop CVD before the age of 60 years (22;23). The pathophysiology of this lipid disorder is still unknown, but the finding of reduced plasma adiponectin levels in patients with FCH supports the hypothesis that a disturbed adipose tissue metabolism may contribute to FCH (17).
Recently tools became available to specifically determine LMW, MMW and HMW forms of adiponectin, and researchers started to focus on these different multimers (3;4;24). In this way it was revealed that the favourable metabolic effects of adiponectin are attributed to the HMW multimer. Plasma levels of the HMW form show a higher correlation with glucose tolerance than total level of adiponectin and this multimer is selectively suppressed in coronary artery disease (CAD) and elevated during weight loss (25-27). Furthermore, Pajvani et al described that the ratio of HMW to total adiponectin, also called the Adiponectin Sensitivity Index (ASI), correlates stronger with insulin sensitivity than just total adiponectin in patients with diabetes type 2 (28).

At the moment no data are present about the adiponectin multimer distribution in patients with FCH. Therefore, the first purpose of this study was to investigate whether a reduced plasma level of total adiponectin in FCH is associated with an altered distribution of the adiponectin multimers. Secondly, we evaluated the associations of adiponectin multimers with the presence of CVD in FCH.

Materials and Methods

Study population

In this study 51 patients with FCH and 51 controls were included. The patients with FCH were derived from a cohort consisting of 37 FCH families (29). The control subjects were obtained from the Nijmegen Biomedical Study (NBS), comprising a random sample of the total population surrounding Nijmegen (30). Patients and controls were matched for age, gender and body mass index (BMI).
Diagnosis of FCH was based on plasma levels of total cholesterol, triglycerides and apoB using the nomogram recently published by Veerkamp et al (29). CVD was defined as presence of peripheral artery disease or history of myocardial infarction, angina pectoris, coronary artery bypass or angioplasty, transient ischemic attack or stroke.
After withdrawal of lipid-lowering medication for four weeks and an overnight fast, blood was drawn by venipuncture. BMI was calculated as body weight (in kilograms) divided by the square of height (in meters). The maximum hip circumference and waist circumference (at the umbilical level) were measured in the late exhalation phase while standing. These two measurements were used to calculate waist-hip ratio (WHR). The study protocol is approved by the ethical committee of the Radboud University Nijmegen Medical Centre and the procedures followed were in accordance with institutional guidelines. All subjects gave written informed consent.

Biochemical analyses

Plasma total cholesterol and total triglycerides were determined by enzymatic, commercially available reagents (catalog number 237574 (Boehringer-Mannheim) and catalog number 6639 (Sera Pak), respectively). HDLc was determined by the polyethylene glycol 6000 method. In the FCH population VLDL was isolated and cholesterol of this fraction was determined as above. LDLc was calculated by subtraction of VLDLc and HDLc from plasma total cholesterol. In the control population LDLc was calculated according to the method of Friedewald. Total plasma apoB concentrations were determined by immunonephelometry. Glucose concentrations were measured in duplicate using the oxidation method (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA).

Intima media thickness (IMT) measurement

Carotid IMT was determined using an AU5 Ultrasound machine (Esaote Biomedica, Genova, Italy) with a 7.5MHz linear-array transducer. Longitudinal images of the distal-most 10 millimetres of both the far wall and the near wall of both common carotid arteries were obtained in the optimal projection (anterolateral, lateral or posterolateral). The sonographer performed the actual measurement of the IMT off-line at the time of the examination, using semi-automatic edge-detection software (M'Ath®Sdt version 2.0, Metris, Argenteuil, France). All measurements were carried out in end-diastole, using the R-wave of a simultaneously recorded ECG as a reference frame. From each frame the mean IMT was calculated over at least 7.5mm of the above mentioned 10mm segment (yielding a quality index of at least 75%). The outcome variable was defined as the mean IMT of the near and far wall of both common carotid arteries (31).

Plasma adiponectin multimer assay

Plasma levels of total, LMW, MMW and HMW adiponectin were determined in duplicate using a commercially available enzyme-linked immunosorbent assay (ELISA) from ALPCO Diagnostics (catalog number 47-ADPH-9755, New Hampshire, USA). This assay is able to quantify total adiponectin, HMW + MMW and HMW directly. The concentrations of LMW and MMW are obtained by subtracting HMW + MMW from total adiponectin and HMW from HMW + MMW, respectively. The Adiponectin Sensitivity Index (ASI) was calculated as the percentage of HMW from total adiponectin (28). The LMW/HMW ratio was calculated as the ratio of LMW to HMW adiponectin. Inter-assay and intra-assay coefficients of variance were 2.7% and 1.3% for total adiponectin, 1.5% and 2.1% for HMW adiponectin, and 11.1% and 2.4% for HMW + MMW, respectively.

Statistical analysis

Continuous variables are expressed as mean ± SD unless otherwise indicated. Variables showing skewed distribution were logarithmically transformed before the analyses. Student's unpaired t-test was used to assess statistical significance of differences observed between patients with FCH and controls. Two-tailed *P*-values less than 0.05 were considered significant. All statistical analyses were performed with the SPSS 14.0 software package.

Results

Characteristics

The anthropometric and metabolic characteristics of the patients with FCH and controls are presented in table 1. Compared to control subjects, patients with FCH

Table 1

Characteristics of patients with familial combined hyperlidemia and controls

Characteristics	FCH patients			Controls		
	All	Men	Women	All	Men	Women
N	51	22 (43%)	29 (57%)	51	22 (43%)	29 (57%)
Age (years)	63.9 (8.0)	62.5 (7.7)	65 (8.2)	62.8 (6.6)	61.6 (6.9)	63.7 (6.3)
BMI (kg/m^2)	28.5 (3.4)	27.9 (3.0)	28.9 (3.6)	28.4 (3.3)	27.8 (2.9)	28.9 (3.6)
WHR	0.93 (0.06)	0.98 (0.04)‡	0.89 (0.05)	0.90 (0.08)	0.96 (0.07)‡	0.85 (0.06)
TC (mmol/L)	7.4 (1.2)*	7.2 (1.2)	7.5 (1.1)	6.3 (1.3)	5.9 (0.9)‡	6.6 (1.4)
TG (mmol/L)	3.7 (2.0)*	4.4 (2.5)	3.3 (1.5)	1.7 (0.8)	1.8 (1.0)	1.6 (0.7)
HDL-c (mmol/L)	1.1 (0.3)*	1.1 (0.23)‡	1.2 (0.24)	1.4 (0.4)	1.3 (0.3)	1.4 (0.4)
LDL-c (mmol/L)	4.4 (1.2)	3.9 (1.1)‡	4.8 (1.2)	4.3 (1.1)	3.9 (0.8)‡	4.5 (1.3)
ApoB (mg/L)	1481 (245)*	1421 (176)	1528 (281)	1127 (268)	1105 (240)	1151 (301)
Glucose (mmol/L)	5.5 (0.9)	5.7 (1.0)	5.3 (0.8)	5.6 (1.1)	5.7 (1.3)	5.6 (0.9)
IMT	0.86 (0.13)	0.90 (0.14)	0.83 (0.11)	0.86 (0.13)	0.87 (0.14)	0.86 (013)
CVD	22 (43%)*	11	11	9 (18%)	5	4

Data are presented as means ± SD; CVD data is presented as number (%). TG, glucose and IMT data are skewed distributed. *, *P*-value<0.05, compared to controls; ‡, *P*-value<0.05, compared to females within the same subgroup. BMI, body mass index; WHR, waist-to-hip ratio; TC, total cholesterol; TG, triglyceride; ApoB, apolipoprotein B; IMT, intima media thickness; CVD, cardiovascular disease.

showed significantly higher levels of TC, TG and apoB. HDLc level was significantly decreased in the FCH population.
Mean WHR tended to be somewhat higher in FCH subjects than in controls, but this difference was not significant. However, WHR was higher and HDLc and LDLc lower in males compared to females, both in patients with FCH and in controls. Plasma glucose was not different between patients with FCH and controls. Mean IMT value was 0.86mm in both groups. The incidence of CVD was higher in patients with FCH than in control subjects (43% vs 18%, *P*-value<0.05).

Levels of total adiponectin and the different multimers in FCH

In both FCH and control group, we observed higher levels of adiponectin in females compared to males (Table 2). Because of this sexual dimorphism, we analyzed the data stratified by gender. Mean total adiponectin levels were lower in FCH patients compared to control subjects, but the difference reached statistical significance for females only. This decrease in total adiponectin in FCH was associated with reduced levels of HMW and LMW adiponectin, again reaching statistical significance in females only. The level of MMW adiponectin did not differ between patients with FCH and controls (Table 2).
Significant correlation was observed with WHR for total adiponectin (controls r= -0.49, FCH r= -0.54), HMW (controls r= -0.47, FCH r= -0.54), MMW (controls r= -0.38, FCH r= -0.46), and LMW (controls r= -0.39, FCH r= -0.30). Total plasma adiponectin, HMW- and MMW adiponectin also significantly correlated with HDLc within FCH patients (total adiponectin r= -0.30, HMW r= 0.28, MMW r= 0.35). Similar correlations of total adiponectin and its multimers with HDLc were found within control subjects. Significant correlation of adiponectin levels with plasma glucose was seen only in the patients with FCH and only for total adiponectin (r= -0.38), HMW (r= -0.36) and MMW (r= -0.36). Total adiponectin and its multimers did not correlate with IMT, neither in patients with FCH nor in controls (data not shown).
Besides the gender specificity in total plasma adiponectin level and its multimers, adiponectin multimer distribution was also different between males and females (Figure 1). Male subjects showed lower ASI and higher LMW/HMW adiponectin ratio compared to females in both patients with FCH and control subjects, reaching statistical significance in control subjects only (ASI: 36.7 ± 11.3% and 42.1 ± 9.8% for FCH males and females, respectively, and 36.4 ± 11.2% and 45.6 ± 11.0% for control males and females, respectively; LMW/HMW adiponectin ratio: 1.3 ± 0.8 and 0.9 ± 0.7 for FCH males and females, respectively, and 1.4 ± 0.7 and 0.9 ± 0.7 for control males and females, respectively). However, no differences were observed in the adiponectin multimer distribution between the patients with FCH and the control subjects (Figure 1).

Table 2

Levels of total plasma adiponectin and its multimers in patients with FCH and controls

	FCH patients			Controls		
	All	Men	Women	All	Men	Women
Total adiponectin (µg/ml)	4.8 (2.3)*	3.3 (1.2)†	5.9 (2.3)‡	6.2 (3.1)	4.1 (1.7)†	7.7 (3.0)
HMW (µg/ml)	2.0 (1.2)*	1.2 (0.6)†	2.6 (1.3)‡	2.8 (1.9)	1.5 (0.9)†	3.7 (2.0)
MMW (µg/ml)	1.1 (0.7)	0.7 (0.3)†	1.4 (0.7)	1.2 (0.7)	0.8 (0.4)†	1.5 (0.8)
LMW (µg/ml)	1.7 (0.8)*	1.4 (0.7)	1.9 (0.9)‡	2.2 (1.0)	1.7 (0.7)†	2.6 (1.0)

Data are presented as means ± SD; Total adiponectin, HMW, MMW and LMW data are skewed distributed. *, P-value<0.05, compared to controls; †, P-value<0.05, compared to females within the same subgroup; ‡ P-value<0.05, compared to female controls. HMW, high molecular weight protein; MMW, middle molecular weight adiponectin; LMW, low molecular weight adiponectin.

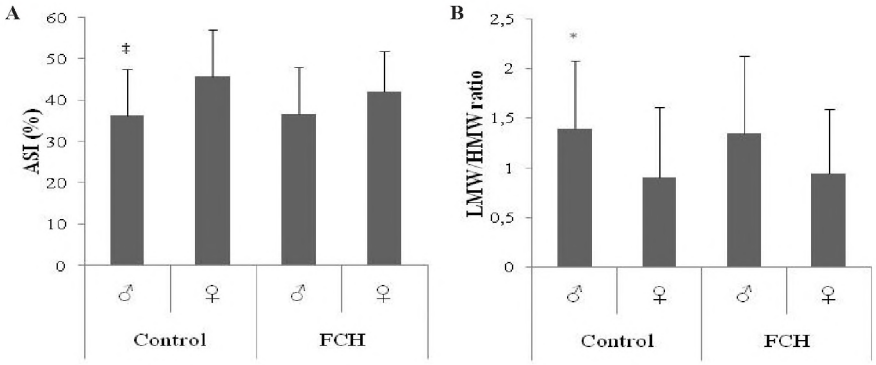

Figure 1 Adiponectin sensitivity index (ASI), reflected by percentage HMW adiponectin to total adiponectin (A) and ratio of LMW and HMW adiponectin in patients with FCH and controls, stratified by gender (B). * *P*-value<0.01, ‡ *P*-value<0.05, compared to females of the control group.

Adiponectin multimer distribution and CVD

No difference was observed between mean total plasma adiponectin of patients with FCH with or without CVD stratified by gender (Figure 2a). We did not compare adiponectin levels of controls with and without CVD, because the number of control subjects with CVD was too low (5 males and 4 females). However, within the FCH population, women with CVD exhibited significantly lower ASI (34.2 ± 10.1% vs 46.0 ± 7.1%) and higher LMW/HMW ratio (1.5 ± 0.8 vs 0.7 ± 0.3) compared to women without CVD (Figure 2b and 2c). Both these ratios were not different in male patients with FCH with and without CVD.

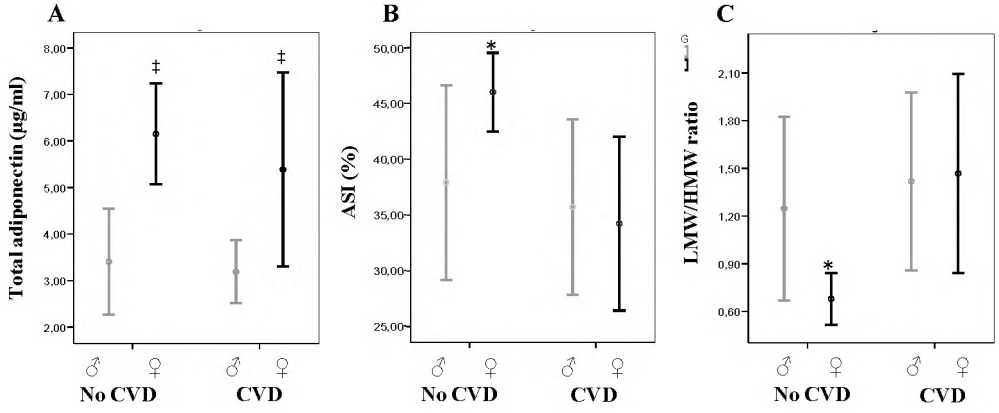

Figure 2 Plasma levels of total adiponectin (A), adiponectin sensitivity index (ASI), reflected by percentage HMW adiponectin to total adiponectin (B) and ratio of LMW and HMW adiponectin in FCH patients with and without CVD, stratified by gender (C). * *P*-value<0.05, compared to FCH females with CVD, ‡ *P*-value<0.05, compared to FCH males with or without CVD.

Discussion

In this study, we show that patients with FCH have a reduced level of total adiponectin, but their adiponectin multimer distribution does not differ from age-, gender-, and BMI- matched controls. However, FCH females with CVD have a less favorable adiponectin multimer distribution with significant lower ASI and higher LMW/HMW adiponectin ratio than FCH females without CVD. Consequently, the profile of adiponectin multimers in FCH females with CVD reflects a more atherogenic multimer distribution.

Like earlier studies, our study shows a clear sexual dimorphism of adiponectin levels in the control population (6). Females have higher total plasma adiponectin than males, and this difference is due to elevated levels of all multimers, but HMW multimer in particular (28;32). As a result of these differences, ASI is higher and LMW/HMW ratio is lower in females compared to males. Gonadal steroids are presumed to be involved in the gender-related difference in adiponectin levels. Testosterone was previously shown to selectively inhibit the secretion of HMW adiponectin (32), and plasma estradiol concentration was shown to negatively correlate with plasma total adiponectin concentration in postmenopausal women (6;33). This effect of testosterone on HMW adiponectin production was hypothesized to partly explain the higher risk of CVD in males. Lara-Castro et al. demonstrated that the HMW adiponectin multimer exhibits close associations with insulin sensitivity, high concentrations of less atherogenic LDL and more cardioprotective HDL (34). In the present study we observed significant correlations of HMW and MMW adiponectin with HDLc and with plasma glucose. These features might contribute to the increased CVD risk in subjects having decreased relative amounts of the HMW adiponectin multimer. Consistent with this, cross-sectional studies have also demonstrated the selective reduction of the HMW adiponectin multimer in type 2 diabetes (25), in CVD (27) and in the metabolic syndrome (35).

Concurrent with the reduction of the relative amount of the HMW multimer of adiponectin we show that in a subset of FCH females, the protective effect of HMW adiponectin against CVD diminishes and CVD risk may rise. Previously, in cross-sectional studies HMW adiponectin has consistently been shown to be a better marker than total adiponectin in the prediction of insulin resistance and the metabolic syndrome (34), endothelial dysfunction (36), and type 2 diabetes (37). In addition, plasma HMW adiponectin was reported to serve as a marker for severity of CAD (38). With respect to prediction of future cardiovascular events, reports, however, are rather contradictory. Inoue et al. showed that plasma HMW adiponectin levels may also predict future CVD events in patients with CAD (38). In contrast, others (39;40), who measured only total adiponectin, reported that high adiponectin level was associated with increased total mortality in patients with chronic heart failure and with recurrence of cardiovascular events in patients who had a recent clinical manifestation of vascular

disease. Poor physical condition may have confounded the outcome of the latter two studies. Patients with chronic heart failure experience weight loss (wasting) due to increased resting energy expenditure and low-grade chronic inflammation precedes recurrence of vascular events. The FCH patients included in the present study were in good physical condition, suggesting that physical condition may not have confounded the analyses.

It has often been shown that visceral adiposity is an independent negative predictor of adiponectin. In addition, Lara-Castro et al showed that this close association of plasma total adiponectin with reduced abdominal fat, is attributed primarily to the HMW adiponectin multimer (34). Therefore, the reduced levels of HMW adiponectin found in the present study in a subset of FCH females may be related to increased visceral adiposity. An increase in visceral adiposity may be associated with adipocyte hypertrophy and lead to less functional adipocytes and altered adipokine production. Consistent with this, we did find a significant inverse correlation of HMW adiponectin with WHR, which is surrogate marker of visceral adiposity.

A limitation of this study is that the cross-sectional design limits inferences about causality. Furthermore, the age of the subjects ranged from 50 to 70 years. As a consequence, most females included in the study were postmenopausal and may have relatively high adiponectin levels due to reduced estradiol concentration. Moreover, due to an impairment in renal function, age is positively associated with plasma adiponectin levels. Unfortunately, no data on renal function of the present population are available. For these reasons our results cannot be generalized to younger subjects.

In conclusion, despite reduced total adiponectin, FCH males show normal adiponectin multimer distribution. However, due to a more pronounced reduction of the HMW multimer in FCH females with CVD, these females have a more atherogenic adiponectin multimer distribution with decreased ASI and increased LMW/HMW adiponectin ratio.

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Chapter 3

Pioglitazone treatment enlarges subcutaneous adipocytes in insulin-resistant patients

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Abstract

Context: Obesity-related insulin resistance is associated with an increase in adipocyte size. In rodent models, treatment with the insulin-sensitizers thiazolidinediones (TZDs) leads to the appearance of small, insulin sensitive adipocytes. Whether such TZD-dependent morphological changes occur in adipose tissue of insulin resistant patients is unclear.

Objective: To study the effects of treatment with the TZD pioglitazone on subcutaneous adipose tissue morphology and function in insulin resistant subjects.

Design: Placebo-controlled, randomized cross-over study.

Setting: University Medical Centre.

Patients: Twelve adult patients with congenital adrenal hyperplasia (CAH) characterized by insulin resistance were included in this study.

Intervention: After a 4-week run-in phase, patients were treated with pioglitazone (45mg/d) followed by placebo, each for 16 weeks or vice versa.

Main Outcome Measures: After both placebo and pioglitazone treatment, insulin sensitivity was determined by hyperinsulinemic euglycemic clamp and abdominal subcutaneous adipose tissue was obtained to measure adipocyte cell surface and expression of genes involved in glucose uptake and inflammation.

Results: Pioglitazone treatment significantly improved the insulin sensitivity index (placebo: 0.35 ± 0.16μmol/kg/min/mU/L; pioglitazone 0.53 ± 0.16μmol/kg/min/mU/L, *P*-value<0.001) and increased mRNA expression levels of adiponectin and GLUT-4 in adipose tissue. The increase in insulin sensitivity was accompanied by a significant enlargement of the subcutaneous adipocyte cell surface (placebo: 2323 ± 725μm^2; pioglitazone 2821 ± 885μm^2, *P*-value=0.03).

Conclusions: In the human situation, treatment of insulin resistant subjects with pioglitazone, improves insulin sensitivity while at the same time subcutaneous adipocyte cell surface increases.

Introduction

Obesity is associated with an abnormal secretion pattern of adipocytes and increased infiltration of macrophages into adipose tissue (1;2). Recent studies have shown that the increased risk for obese subjects to develop insulin resistance and type 2 diabetes mellitus (T2DM) is associated with increased subcutaneous abdominal adipocyte surface (3;4). Large adipocytes are relatively insulin-insensitive and have an increased basal lipolysis and an altered secretion of adipokines (5;6). In addition, hypertrophic adipocytes promote the infiltration of macrophages into adipose tissue leading to enhanced production of pro-inflammatory cytokines that contribute to the development of insulin insensitivity (7). It has therefore been assumed that the presence of large adipocytes may drive the development of insulin resistance.
Thiazolidinediones (TZDs) are an insulin-sensitizing class of pharmacological compounds widely used for treatment of T2DM, which exhibit their insulin-sensitizing effects by decreasing visceral fat content, enhancing insulin action and improving glucose homeostasis (8). At the same time, TZDs increase the subcutaneous fat depot (9). TZDs are ligands for peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear transcription factor highly expressed in adipose tissue (10). One of the adipose tissue-specific effects of TZDs, mainly described in rodent studies, is the stimulation of adipocyte differentiation and apoptosis of large adipocytes, resulting in an increased population of small, more insulin sensitive adipocytes (11). This suggests that improvement in glucose homeostasis by TZD treatment will be accompanied by changes in adipocyte size.
In the present study, we investigated the effects of pioglitazone treatment on insulin sensitivity, subcutaneous adipocyte surface and expression of genes involved in glucose uptake and inflammation, in insulin resistant subjects.

Materials and Methods

Study population
Twelve patients with genetically-characterized, "classical", congenital adrenal hyperplasia (CAH) on a stable corticosteroid dose were studied in a randomized cross-over design. Based on their underlying condition and chronic treatment, this group of patients is characterized by insulin resistance (12). After a 4 week run-in phase, patients were randomized to treatments with placebo and pioglitazone (45mg/d) in a blinded fashion for 16 weeks, or vice versa. At the end of each treatment period, subcutaneous adipose tissue biopsies were obtained under local anesthesia by needle biopsies 6-10cm lateral to the umbilicus. Samples were taken after an overnight fast. Fasting plasma adiponectin, leptin, insulin and glucose were determined and insulin sensitivity index (ISI) was assessed. Finally, the percentage of trunk fat was determined.

The ethical committee of the Radboud University Nijmegen Medical Centre approved the study protocol and the procedures followed were in accordance with institutional guidelines. All subjects gave written informed consent.

Adipocyte surface

Morphometry of individual fat cells was assessed in a blinded fashion using digital image analyses as described recently (13). In short, after biopsy, adipose tissue was immediately fixed in paraformaldehyde and embedded in paraffin. Subsequently, paraffin slides were stained with heamatoxylin-eosin. Inasmuch our method to measure adipocyte size requires intact cell membranes, we initially optimized the thickness of our sections. Microscopic observation revealed that adipose tissue samples cut into sections < 8μm showed ruptured cells. To avoid multilayered cells, we used 10μm sections for the analysis. For each subject, surface of all fat cells in 4–7 microscopic fields of view were measured. On average, 250 fat cells were measured per specimen (range 150–350).

RNA isolation and real time PCR analysis

Total RNA was extracted from subcutaneous adipose tissue using TRIzol reagent (Invitrogen, Carlstad, USA). RNA concentration was determined using the NanoDrop™ (NanoDrop Technologies, Wilmington, USA). cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio–Rad Laboratories, Hercules, CA). Real-time PCR was performed using Power-SYBR® Green master mix and the 7300 Real-Time PCR system (Applied Biosystem, Warrington, UK). Expression of genes was normalized to β2M gene expression levels. Primer sequences are available upon request.

Insulin sensitivity and body fat distribution

Insulin sensitivity was assessed by a hyperinsulinemic euglycemic clamp and expressed as glucose disposal rate (Rd) and ISI essentially as described previously (14). Total-body Dual-energy X-ray absorptiometry (DEXA) scanning was performed using a Hologic QDR 4500 densitometer to determine indirectly the percentage of trunk fat mass by dividing the absolute fat mass of the trunk by the total mass of the trunk.

Biochemical analyses

Glucose concentrations were measured using the oxidation method (Beckman®, Glucose Analyser2, Beckman Instruments Inc., Fullerton, CA 92634, USA). Plasma concentrations of leptin and total adiponectin were determined using enzyme-linked immunosorbent assays (R&D Systems, Minneapolis). The inter-assay and intra-assay coefficient of variation were 6.5% and 3.5% for total adiponectin and 4.4% and 3.2% for leptin.

Statistical analyses

Variables are expressed as means ± SD. Differences in adipocyte surface after placebo and pioglitazone treatment were studied using one-way univariate analysis of variance. Students' paired t-test was used to analyze statistical significance of treatment differences of other variables. Two-tailed *P*-values less than 0.05 were considered significant. Tukey honestly significant difference post-hoc testing was applied for multiple comparison testing. All statistics were performed using SPSS software (SPSS 16.0 Inc, Chicago, Il).

Results

Study population characteristics

A total of 12 patients (five men, seven women; age 36 ± 9yr; BMI 26.9 ± 4.7kg/m^2) completed the study. Mean fasting plasma insulin, glucose, triglycerides, and total cholesterol levels after placebo were 8.8 ± 5.8mU/L, 5.0 ± 0.2mmol/L, 0.96 ± 0.3mmol/L and 4.53 ± 0.7mmol/L, respectively. The cell surface of subcutaneous adipocytes in our study population showed large variation. Whereas the mean subcutaneous adipocyte surface was 2323 ± 725µm^2, clear inter-individual differences were observed (Figure 1A and B).

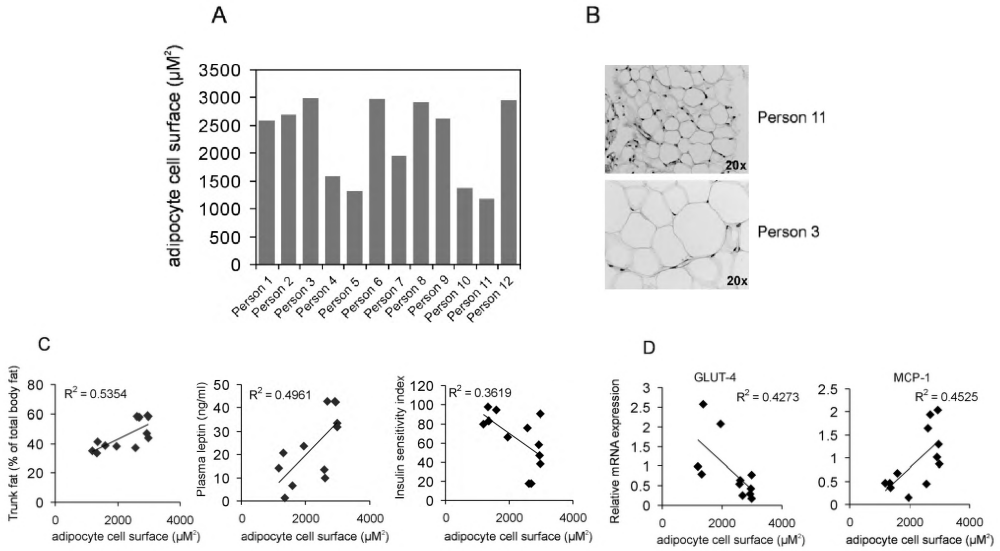

Figure 1 **Characteristics of subcutaneous adipocytes after placebo.** A: Morphometry of fat cells assessed by digital image analysis. B: Heamatoxylin-eosin staining images of subcutaneous adipose tissue of two subjects. C: Pearson correlation of mean adipocyte surface with percentage of trunk fat, plasma leptin level, insulin sensitivity index, and (D) gene expression levels of Glut-4 and MCP -1.

Interestingly, adipocyte cell surface was positively correlated with the percentage of trunk fat (r=0.73; *P*-value=0.01) and plasma leptin levels (r=0.70; *P*-value=0.01) and negatively correlated with the ISI (r=-0.60; *P*-value=0.04) (Figure 1C). In addition, adipocyte surface correlated negatively with glucose transporter 4 (Glut-4) mRNA expression (r= -0.65; *P*-value=0.03) and positively with the mRNA expression level of the inflammatory marker monocyte chemoattractant protein-1 (MCP-1) in the adipose tissue (r=0.57; *P*-value=0.05) (Figure 1D). Noticeably, adjustment for BMI did not significantly alter the correlations that were observed between the different variables and adipocyte surface.

Effects of pioglitazone treatment

After 16 weeks of pioglitazone treatment, body weight and BMI had increased (by 2.0 ± 3.3kg and 0.7 ± 1.1kg/m^2; *P*-value=0.06 for both), but no difference was observed in mean trunk fat percentage. Insulin sensitivity improved significantly after pioglitazone as reflected by ISI (placebo: 0.35 ± 0.16; pioglitazone: 0.53 ± 0.16µmol/kg/min/mU/L); *P*-value<0.001). The ISI was based on Rd (placebo: 28.5 ± 11.6µmol/kg/min; pioglitazone 38.9 ± 11.0µmol/kg/min, *P*-value<0.001) and the insulin level at 120 minutes (placebo: 87.5 ± 22.9mU/L; pioglitazone 76.3 ± 13.8mU/L; *P*-value=0.03). Subcutaneous adipocyte surface increased significantly (by 497 ± 625µm^2; *P*-value=0.03) following pioglitazone treatment: 8 out of 11 patients showed larger adipocytes (Figure 2A and B). Pioglitazone had no significant effect on fasting plasma insulin (7.9 ± 4.1mU/L after pioglitazone), glucose (5.1 ± 0.5mmol/L after pioglitazone), adiponectin (placebo: 5.4 ± 3.9µg/mL; pioglitazone: 6.1 ± 4.7µg/mL) and leptin levels (placebo: 23.6 ± 14.9ng/mL; pioglitazone 22.9 ± 16.3ng/mL). qPCR analysis revealed increases in expression of adiponectin (*P*-value<0.01) and GLUT-4 (*P*-value=0.10) mRNA in adipose tissue after pioglitazone treatment. MCP-1 mRNA expression appeared lower after pioglitazone treatment, but the differences were not statistically significant (Figure 2C).

After pioglitazone treatment, the negative correlation between subcutaneous adipocyte surface and ISI was slightly weaker (r= -0.58; *P*-value=0.05), and the negative correlation between GLUT-4 and subcutaneous adipocyte surface was no longer significant (r= -0.42; *P*-value=0.2). Correlations between subcutaneous adipocyte surface and the percentage of trunk fat, plasma leptin levels, and MCP-1 gene expression were dramatically changed after pioglitazone treatment. In contrast to placebo treatment, the percentage trunk fat and plasma leptin levels were no longer positively correlated with subcutaneous adipocyte surface. In addition, the positive correlation of MCP-1 gene expression levels and adipocyte surface observed after placebo treatment, was lost after pioglitazone treatment. Apparently, enlarged adipocyte surface no longer predicts metabolic abnormalities and adipose tissue functioning after pioglitazone treatment in this study population.

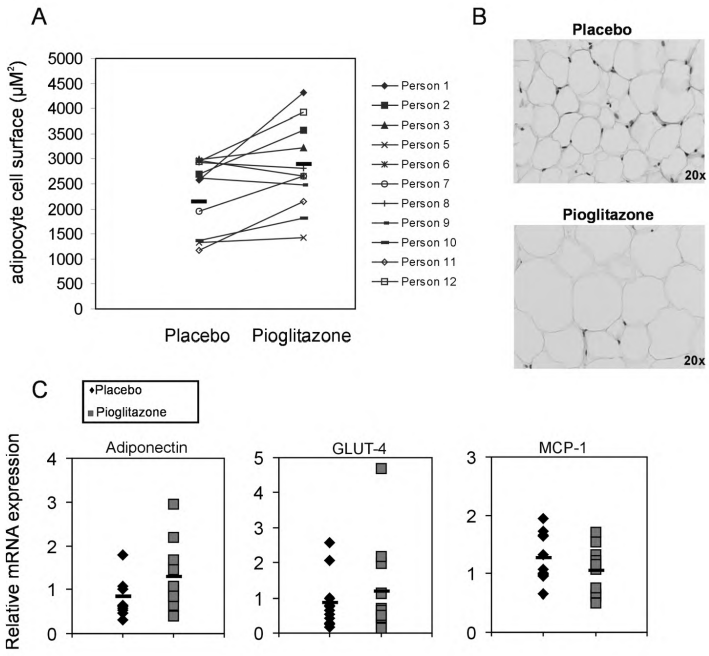

Figure 2 **Characteristics of subcutaneous adipocytes after 16 weeks of pioglitazone treatment.** A: Individual changes of mean subcutaneous adipocyte cell surface following pioglitazone treatment. B: Representative heamatoxylin-eosin staining images of subcutaneous adipose tissue after placebo and after pioglitazone treatment. C: Individual adipose tissue mRNA expression levels of adiponectin, Glut-4 and MCP-1 after both treatments. Person 4 is missing since no biopsy was taken after pioglitazone treatment. Black dashes represent mean values.

Discussion

In the present study we describe the effect of pioglitazone on subcutaneous adipose tissue morphology, gene expression levels and insulin sensitivity in a group of insulin resistant patients. Although adipocyte hypertrophy was associated with indices of insulin resistance, pioglitazone treatment resulted in an enlargement of subcutaneous adipocytes in parallel with an improvement of systemic insulin sensitivity.
The positive association between obesity-induced adipocyte hypertrophy and the development of insulin resistance and T2DM has been reported before (4). The development of hypertrophic adipocytes is partly explained by the impaired ability to differentiate new fat cells, resulting in enlargement of existing adipocytes (15). Enlarged adipocytes are characterized by a lower insulin mediated glucose disposal, disturbances in lipid mobilization, and secretion of higher levels of leptin compared with smaller adipocytes (3;16). In line with these detrimental effects of adipocyte hypertrophy, our data show that after placebo enlarged subcutaneous adipocyte surface correlates with

elevated levels of circulating leptin, low insulin sensitivity, and lower GLUT-4 mRNA expression, an essential glucose uptake transporter. Adipocyte surface also positively correlates with secretion of pro-inflammatory adipokines (17). In our study, the MCP-1 pro-inflammatory gene expression positively correlated with subcutaneous adipocyte surface, suggesting that enlarged adipocytes have increased secretion levels of MCP-1 (17). Elevated MCP-1 levels might also be indicative for increased accumulation of macrophages in subcutaneous adipose tissue that promote the development of insulin resistance. Altogether, our observations suggest that subcutaneous adipocyte hypertrophy is indicative for unfavorable metabolic functioning of adipose tissue after placebo treatment.

In agreement with previously published studies, treatment with pioglitazone significantly improved insulin sensitivity in our study population despite an increase in body weight (18). Since PPAR-γ efficiently binds TZDs and is abundantly expressed in adipose tissue, pioglitazone is thought to primarily target the adipose tissue where it promotes the development of small adipocytes characterized by an improvement in metabolic function. However, evidence in support of this mechanism of action has mainly been obtained from rodent studies (11). The number of studies that have investigated subcutaneous adipocyte surface in response to TZD treatment in humans is limited and has revealed ambiguous results. So far, studies have reported no change or an increase in the number of small adipocytes together with an improvement of insulin sensitivity after TZD treatment (19;20), although a trend towards fat cell volume increase was also observed (21). In the present study we observed a clear increase in subcutaneous adipocyte surface by pioglitazone together with an improvement of whole body insulin sensitivity. These divergent results may be explained by differences in methodology. Previous studies have measured adipocyte surface using isolated adipocytes obtained after collagenase digestion and subsequent centrifugation. This treatment usually results in breakage of large cells leading to an unrepresentative sample of the original cell population with underestimation of large cells (22). In the present study, immediate fixation of adipose tissue biopsies after isolation warranted accurate estimation of adipocyte surfaces.

Importantly, positive correlations between adipocyte surface and plasma leptin, percentage trunk fat, and MCP-1 gene expression levels, markers indicative for insulin resistance and adipose tissue dysfunctioning, disappeared after pioglitazone-induced enlargement of adipocytes. These findings suggest that pioglitazone-induced adipocyte surface enlargement has not a detrimental but a beneficial effect on adipose tissue metabolism. Hypothetically, the pioglitazone-induced adipocyte hypertrophy might result from redistribution of visceral and ectopic fat towards subcutaneous storage ultimately leading to smaller visceral adipocytes, though enlarged subcutaneous adipocytes. Since no visceral fat biopsies were taken, no data is available to support this hypothesis.

This study was performed in a group of adult patients with CAH on stable corticoste-

roid treatment. These patients are characterized by insulin resistance (12), and were used in this study as a human "model" for obesity-associated insulin resistance. The advantage of this approach is that pioglitazone treatment will not lead to changes in glucose levels and hence the "pure" – not confounded by changes in glycemic level – effect of the TZD on fat metabolism can be studied. The improvement of insulin sensitivity suggests a normal response to pioglitazone in CAH patients and affirms the applicability of the model. However, we cannot exclude that other human models of insulin resistance would show different responses.

In conclusion, our data, obtained from human adipose tissue samples, confirm that increased subcutaneous adipocyte surface correlates with indices of insulin resistance at the whole body and the cellular level. Pioglitazone treatment enlarges adipose tissue and increases adipocyte surface, while at the same time improving the systemic insulin sensitivity.

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Chapter 4

IL-18 protects against the development of high fat diet-induced insulin resistance

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Submitted

Abstract

Introduction: In parallel with the development of insulin resistance, obese individuals display a chronic low grade inflammation characterized by an increased activity of numerous pro-inflammatory cytokines. Cytokines appear to make divergent contributions since some facilitate and others seemingly inhibit the development of obesity-induced insulin resistance. Although IL-18, member of the IL-1 family of cytokines, has been positively correlated with the presence of obesity and insulin resistance, IL-18 has also been shown to suppress food intake, improve insulin sensitivity and prevent the development of obesity. Therefore we hypothesized that IL-18 protects against the development of high fat diet-induced insulin resistance.

Materials and Methods: To test our hypothesis, wild-type and IL-18-/- animals were fed a low fat diet (LFD) or high fat diet (HFD) for 16 weeks to induce obesity and insulin resistance.

Results: Gene expression analysis revealed that IL-18 is highly expressed in liver and white adipose tissue. Although a HFD induced weight gain and hepatic steatosis to a similar extend in both genotypes, insulin sensitivity tests revealed that IL-18-/- mice fed the HFD were more insulin resistant compared to wild-type animals. In parallel, IL-18-/- animals displayed lower plasma adiponectin levels, elevated plasma VLDL triglycerides and higher serum ALT levels. Treatment of animals with recombinant IL-18 improved hepatic insulin sensitivity as determined by pAKT levels. Surprisingly, adipose tissue macrophage influx was lower in HFD fed IL-18-/- animals as compared to wild-type controls concurrent with lower adipose tissue levels of the chemokine MCP-1. However, gene expression analysis revealed higher levels of IL-6 and lower expression levels of IL-1 receptor antagonist in adipose tissue of HFD-fed IL-18-/- animals.

Conclusions: IL-18 determines insulin sensitivity independently of the development of HFD-induced obesity, hepatic steatosis and influx of macrophages into adipose tissue.

Introduction

Obesity fuels the development of a chronic low grade inflammation illustrated by increased plasma concentrations of pro-inflammatory mediators (1). It is generally believed that obesity-induced inflammation originates from expanding adipose tissue that secretes a wide variety of proteins named adipokines (2). During the development of obesity, adipose tissue undergoes morphological changes including adipocyte hypertrophy accompanied by the infiltration of macrophages (3). This leads to a more pro-inflammatory secretion profile of adipose tissue from obese individuals compared to lean subjects (4). In addition to changes of the adipose tissue, the liver also undergoes morphological changes during the development of obesity. Fat accumulation in liver is a common phenomenon in obese individuals and can eventually result in the development of hepatic inflammation, enhanced plasma ALT levels and increased production of pro-inflammatory proteins (5).
Although it is well established that obesity leads to a state of chronic low grade inflammation and insulin resistance, various cytokines make divergent contributions to this process. Whereas several cytokines positively contribute to the development of obesity and insulin resistance, others appear to inhibit this pathophysiological process. Tumor necrosis factor (TNF)-α is able to induce insulin resistance (6) and anti-TNFα treatment of insulin resistant individuals improves insulin sensitivity (7;8). Recent work in IL-18-/- animals revealed an important role of IL-18 in metabolic processes that modulate energy balance and insulin sensitivity (9;10). IL-18, part of the IL-1 cytokine family, has initially been identified as a potent inducer of interferon gamma (IFN)-γ secretion participating in inflammatory processes (11). Unlike other members of the IL-1 cytokine family that are induced during acute inflammation, IL-18 is constitutively expressed (12). Despite these pro-inflammatory capacities, IL-18 seems to protect against the development of obesity and insulin resistance. In contrast, several lines of evidence hint at a role for IL-18 in inducing obesity-related complications since circulating levels of IL-18 are increased during obesity individuals and have been positively correlated to the development of the metabolic syndrome (13;14). Additionally, weight loss has been shown to reduce circulating levels of IL-18 (15). However, it can be envisioned that elevated IL-18 plasma concentrations are the result of compensatory mechanisms due to a reduced response to IL-18 similar to the development of hyperinsulinemia and hyperleptinemia in obese individuals (16).
In this study, we hypothesized that IL-18 is essential for effective insulin signaling during high fat diet. To test our hypothesis, both wild-type and IL-18-/- animals were fed a high fat diet to induce insulin resistance. Our results show that IL-18 controls insulin sensitivity independently of the development of obesity, hepatic steatosis and macrophage influx into the adipose tissue.

Material and methods

Animal studies
IL18-/- mice were generated on a C57Bl/6J background as previously described (17). Age-matched wild type C57Bl/6J mice were used as the control group. Body weight of the mice was measured every month. To induce obesity, animals were fed a high fat diet containing 45 energy% from fat (D12451, Research diets, New Brunswick, USA). Control animals received a low fat diet containing 10 energy% from fat (D12450B, Research diets, New Brunswick, USA).

Insulin tolerance tests
Insulin-tolerance tests were performed as previously described (18). Briefly, the insulin-tolerance tested animals were fasted overnight. Insulin was given intraperitoneally at a dose of 0.75U/kg bodyweight. Blood samples were taken before and 30, 60, 90 or 120 minutes after insulin administration to determine the plasma glucose levels.

RNA isolation and qPCR analysis
RNA from animal tissues was isolated using Trizol Reagent (Invitrogen) following manufacturer's instructions. RNA concentration and purity was determined using a Nanodrop (Thermo Fisher Scientific, USA). 500ng or 1µg of RNA was used for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Real-Time PCR was done with a Power Sybr Green PCR master mix (Applied Biosystems) using a 7300 Real-Time PCR System (Applied Biosystems). Primers sequences are available upon request. Melt curve analysis was included to assure a single PCR product was formed. Values were corrected using the housekeeping gene 36B4.

Histology/immunohistochemistry
For detection of macrophages, an F4/80+ antibody (Serotec, Oxford, UK) was used. Sections were pre-incubated with 20% normal goat serum followed by overnight incubation at 4 °C with the primary antibody diluted 1:150 in PBS/ 1% Bovine Serum Albumin (BSA). After incubation with the primary antibody, a goat anti rat IgG conjugated to horseradish peroxidase (Serotec) was used as secondary antibody. Visualization of the complex was done using 3,3'-diaminobenzidene for 5 minutes. Haematoxylin and Eosin (HE) staining of sections was done using standard protocols to determine white adipose tissue and liver morphometry.

Plasma analysis
Plasma insulin, adiponectin, leptin and adipose tissue MCP-1 levels were measured using commercially available ELISA kits following manufacturer's instructions. The insulin ELISA kit was from Linco, adiponectin, leptin and MCP-1 ELISA kits were

from R&D systems. Plasma glucose, cholesterol and triglyceride levels were determined using commercially available kits from Human. Plasma IL-18 levels were measured using an ELISA kit from Invitrogen following manufacturer's instructions.

Statistical analysis
Statistical significant differences were calculated using a Student's T-test. The cut-off for statistical significance was set at a *P*-value of 0.05 or below.

Results

IL-18 is mainly expressed in liver and adipose tissue
To compare the relative contribution of muscle, liver, and fat to express IL-18, transcription levels were determined by qPCR. As shown in figure 1A, IL-18 is most prominently expressed in liver and adipose tissue. Interestingly, among the IL-1 family members, IL-18 displayed the highest basal gene expression levels suggestive of a different function as compared to other members within the IL-1 family of cytokines (Figure 1B). To identify the cellular source of IL-18, immunohistochemical staining and qPCR analysis of specific fractions of the liver and adipose tissue of wild-type C57/Bl6 animals were performed. As shown in figure 1C, IL-18 protein is predominantly expressed in kupffer cells, yet qPCR analysis revealed gene expression of IL-18 in both hepatocytes and Kupffer cells. Transcriptional and immunohistochemical analysis of the adipose tissue revealed that IL-18 is primarily expressed in adipocytes compared to the stromal vascular fraction (SVF) (Figure 1D).

IL-18-/- animals fed a high fat diet are more prone to the development of insulin resistance
We next set out to study the effect of IL-18 during the development diet-induced obesity and insulin resistance. Interestingly, plasma concentrations of IL-18 were positively correlated with the body mass of mice with varying degrees of adiposity (Figure 2A) suggesting that IL-18 participates in the development of obesity. To explore the effects of IL-18 during the development of obesity, wild-type and IL-18-/- animals were fed a high fat diet (HFD) or a low fat diet (LFD). The HFD induced a comparable weight gain in both IL-18-/- animals and wild-type mice (Figure 2B). Plasma insulin analysis revealed a significant 2-fold increase (P-value<0.01) in wild-type mice compared to IL-18-/- mice fed the LFD (Figure 2C). Although insulin levels tended to be higher in the IL-18-/- fed the HFD compared to their wild-type controls (Figure 2C), results were not significantly different. Whereas plasma leptin levels were increased due to HFD feeding, no differences between both genotypes were observed (Figure 2D). Interestingly, plasma adiponectin levels were significantly lower in HFD fed IL-18-/- animals compared to the wild-type controls (Figure 2E).

Diet-induced changes in plasma glucose (Figure 2F) and cholesterol (Figure 2G) concentrations displayed similar profiles in wild-type and IL-18-/- animals. In contrast, plasma triglyceride (TG) concentrations were significantly higher in IL-18-/- animals independent of the diet interventions (Figure 2G). Detailed analysis of the TG content in the different lipoprotein fractions revealed a significant 2-fold increase in very low-density lipoprotein (VLDL)-TG concentrations in IL-18-/- animals independently of the dietary intervention (Table 1).

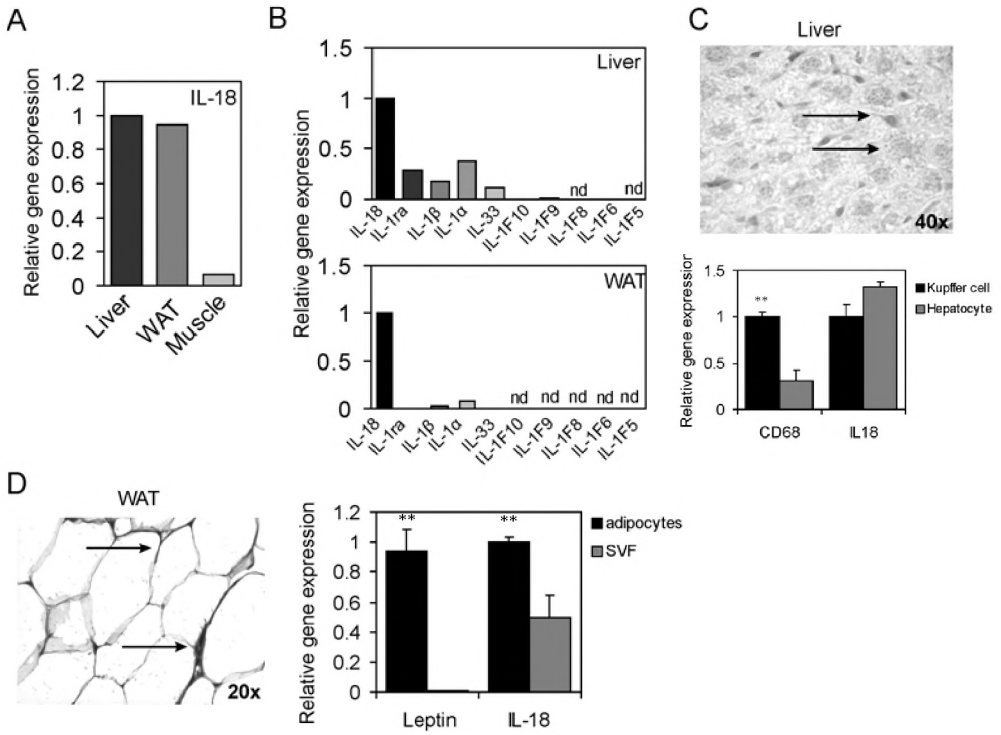

Figure 1 **IL-18 expression in white adipose tissue and liver of C57/Bl6 wild-type animals** A. Gene expression levels of IL-18 determined in liver, white adipose tissue and muscle (*n*=1 per group). B. Expression analysis of all IL-1 family-members in liver and white adipose tissue by qPCR (*n*=1 per group). C. Immunohistochemical staining in liver and qPCR analysis using hepatocytes or kupffer cells of IL-18. The arrows indicated positive staining of cells. Macrophage marker CD68 was used as a positive control to discriminate Kupffer cells from hepatocytes (*n*=10 per group) D. Immunohistochemical staining in white adipose tissue and qPCR analysis using adipocytes or stromal vascular cells of IL-18. Adipocyte marker leptin was used as a positive control to discriminate adipocytes from stromal vascular cells (*n*=10 per group). The arrows indicate positive staining of cells. Data are presented as means ± SEM. **= *P*-value<0.01. nd=not detected.

Table 1

Plasma TG levels in different plasma lipoprotein fractions

Animals	VLDL-TG (mmol/L)	HDL-TG (mmol/L)	LDL-TG (mmol/L)
Wild-type-LFD	0.37 ± 0.16	0.13 ± 0.01	0.09 ± 0.008
Wild-type-HFD	0.24 ± 0.014	0.25 ± 0.029	0.09 ± 0.06
IL-18-/- LFD	0.71 ± 0.212*	0.14 ± 0.02	0.08 ± 0.003
IL-18-/- HFD	0.57 ± 0.074#	0.20 ± 0.01	0.11 ± 0.007

Data are presented as means ± SEM. *, *P*-value<0.05, compared to wild-type animals fed a low fat diet (LFD). #, *P*-value<0.05, compared to wild-type animals fed a high fat diet (HFD). VLDL, very low density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein. (*n*=10 per group).

To determine the effect of IL-18 on HFD-induced insulin resistance, an insulin tolerance test was performed. Whereas HFD-feeding led to the development of insulin resistance in wild-type animals compared to animals fed the LFD (data not shown), depletion of IL-18 aggravated the development of insulin resistance after the HFD intervention (Figure 2H). To counteract the absence of IL-18, we treated IL-18-/- animals with a single dose of PBS or recombinant IL-18 (10µg/ml) 24 hours prior to short term exposure to insulin. Interestingly, pre-treatment with IL-18 led to an enhancement of insulin-induced hepatic pAKT levels as compared to IL-18-/- animals pre-treated with PBS only (Figure 2I), which suggest a direct effect of IL-18 on hepatic insulin sensitivity.

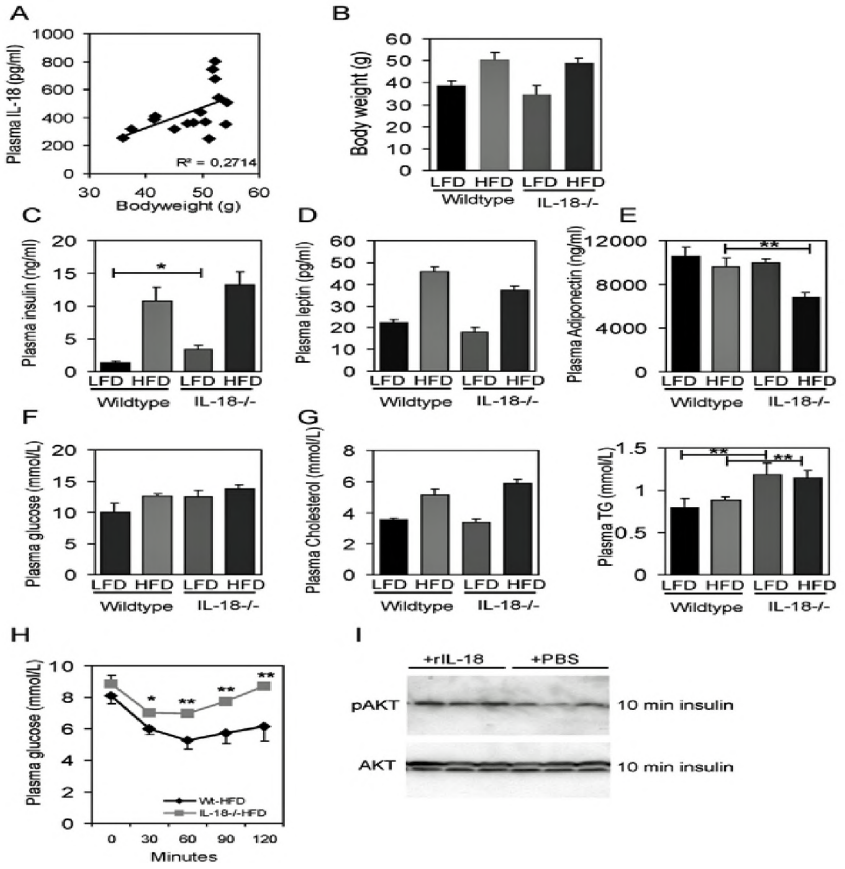

Figure 2 **Absence of IL-18 leads to insulin resistance in animals with diet-induced obesity.** A. Plasma levels of IL-18 correlates positively with body weight (grams) in wild-type mice. B. Body weight of wild-type and IL-18-/- mice fed a high fat diet (HFD) or a low fat diet (LFD) for 16 weeks (*n*=10 per group). C. Plasma levels of insulin, leptin (D), adiponectin (E), glucose (F), cholesterol and triglycerides (G) in wild-type and IL-18-/- mice fed a HFD or a low fat diet (LFD) for 16 weeks (*n*=10 per group). H. Insulin tolerance test in wild-type and IL-18-/- animals fed a HFD (*n*=5 per group).

I. Western blot analysis of phosphorylated AKT and total AKT levels after insulin (0.0225U/mouse) treatment for 10 minutes (n=3 per group). Data are presented as means ± SEM. Asterisks represents statistically significant differences between both genotypes within the same dietary intervention group. * =P-value<0.05, ** = P-value<0.01.

Inasmuch IL-18 has been shown to affect food intake (9;10), we studied gene expression levels of hypothalamic neuropeptides that regulate feeding behaviour. Interestingly, although food intake levels were higher in LFD-fed IL-18-/- animals, HFD-feeding did not result in significant changes in food intake between wild-type and IL-18-/- mice (Figure 3A) suggesting that IL-18-/- animals do not overeat the HFD. Our results correspond with earlier work (10) and imply that IL-18 is involved in determining macronutrient preference. Hypothalamic gene expression levels of the orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AGRP) were significantly increased in HFD-fed IL-18-/- animal (Figure 3B). In contrast, hypocretin gene expression levels were 2 times lower in IL-18-/- mice fed a LFD compared to wild-type control animals (Figure 3B). Anorexigenic neuropeptides proopiomelanocortin (POMC) and cocaine- and amphetamine regulated transcript (CART) did not show any differences in gene expression between wild-type and IL-18-/- animals fed a LFD or HFD (Figure 3C).

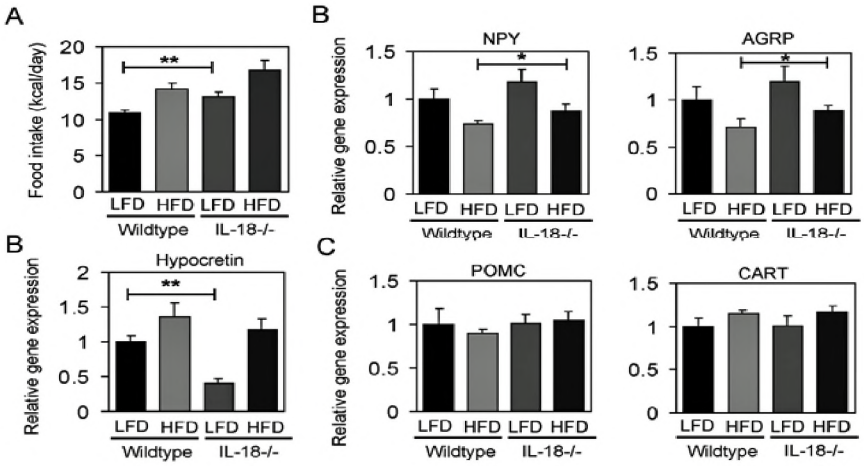

Figure 3 **Role of IL-18 in controlling hypothalamic neuropeptide expression levels**. A. Food intake (kcal/day) of wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group). B. Gene expression levels of the orexigenic neuropeptide genes NPY, AGRP and hypocretin in the hypothalamus of wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group). C. Gene expression levels of the anorexigenic neuropeptide genes POMC and CART in the hypothalamus of wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group). Data are presented as means ± SEM. Asterisks represents statistically significant differences between both genotypes within the same dietary intervention group. * = P-value<0.05, ** = P-value<0.01.

Liver and adipose tissue-specific effects in IL-18-/- animals fed a HFD

Since IL-18 is predominantly expressed in liver and adipose tissue, adipose- and liver-specific effects in IL-18-/- animals during the development of high-fat diet induced obesity were analyzed. First, we examined changes in hepatic morphology, triglyceride storage and gene expression after the diet intervention in wild-type and IL-18-/- animals. Liver morphology, as assessed by HE-staining, revealed a similar degree of hepatic steatosis within the same dietary intervention groups (Figure 4A) that was confirmed by quantification of the hepatic triglyceride levels in wild-type and IL-18-/- mice (Figure 4B). However, plasma alanine transaminase (ALT) showed a significant 2-fold elevation in animals lacking IL-18 fed the HFD compared to the wild-type controls (Figure 4C), suggestive of an increase in liver damage. Noticeably, although HFD significantly induced gene expression levels of multiple liver injury markers including tissue inhibitors of metalloproteinase (TIMP)-1, matrix metalloproteinase (MMP)-12 and α-smooth muscle actin (SMA), no differences were observed between wild-type and IL-18-/- fed the HFD (data not shown).

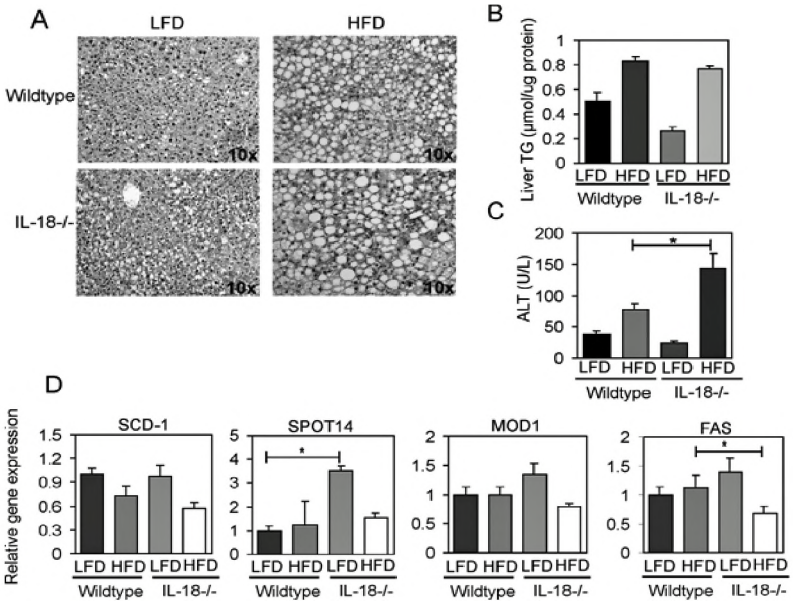

Figure 4 **Liver specific effects of IL-18 in diet-induced obesity.** A. Representative HE staining of liver tissue from wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks. B. Intra-hepatic triglyceride levels of wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group). C. Plasma ALT levels in wild-type and IL-18-/- animals fed a HFD or a low fat diet (LFD) for 16 weeks (n=10 per group). D. Gene expression levels of the lipogenic genes SCD-1, SPOT14, MOD1 and FAS in the liver of wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group).Data are presented as means ± SEM. Asterisks represents statistically significant differences between both genotypes within the same dietary intervention group. * = P-value<0.05.

Since increased levels of hepatic lipogenesis may contribute to the elevated plasma VLDL-TG levels observed in IL-18-/- animals (19), we studied gene expression levels of several lipogenic markers in liver. As shown in figure 4D, gene expression levels of stearoyl-Coenzyme A desaturase (SCD)-1 and mosaic death (MOD)-1 were not significantly different between wild-type and IL-18-/- animals fed either the LFD or HFD. SPOT14 was 3.5 times higher expressed in IL-18-/- mice fed a LFD as compared to the wild-type controls, whereas after HFD feeding fatty acid synthase (FAS) showed a significant decrease in IL-18-/- mice compared to their wild-type control. The HFD intervention did not resulted in differences in adipose tissue mass (data not shown) or adipocyte cell size between wild-type and IL-18-/- animals (Figure 5A). Inasmuch insulin resistance is partly induced by the development of adipose tissue inflammation characterized by the influx of macrophages (20), qPCR and immunohistochemical analysis of the white adipose tissue was performed. Despite an impairment in insulin sensitivity (Figure 2I), analysis of transcriptional macrophage markers (Figure 5C) and immunohistochemical staining using an antibody against F4/80+ (Figure 5B), revealed a significant reduction in macrophages present in white adipose tissue of HFD-fed IL-18-/- animals. The chemokine monocyte chemoattractant protein (MCP)-1 plays an essential role in attracting the macrophages into the tissue. In line with the reduction in macrophage influx into the adipose tissue, adipose tissue levels of MCP-1 were significantly reduced in IL-18-/- animals compared to wild-type animals fed the HFD (Figure 5D).
Despite a reduction in macrophage content of the adipose tissue, gene expression levels of TNFα were not lower in adipose tissue of HFD-fed IL-18-/- animals. However, interleukin (IL)-6 expression levels were increased and levels of the anti-inflammatory protein IL-1 receptor antagonist (IL-1Ra) were reduced in adipose tissue of IL-18-/- animals compared to the wild-type controls after 16 weeks of HFD feeding (Figure 5E).

Discussion

The main finding of this study is that IL-18 controls insulin sensitivity independently of the development of obesity, hepatic steatosis or macrophage influx after 16 weeks of HFD feeding. Moreover, IL-18 appears to be required for effective hepatic insulin signaling as treatment with recombinant IL-18 improved insulin-induced phosphorylation of AKT in vivo.
Our results appear to contradict with previous studies showing that obesity results in an elevated state of inflammation characterized by an increased activity of numerous inflammatory cytokines including TNFα, IL-6 and IL-18 contributing to the development of insulin resistance (21). Our findings support the hypothesis that pro-inflammatory cytokines do not unequivocally induce insulin resistance exemplified by the

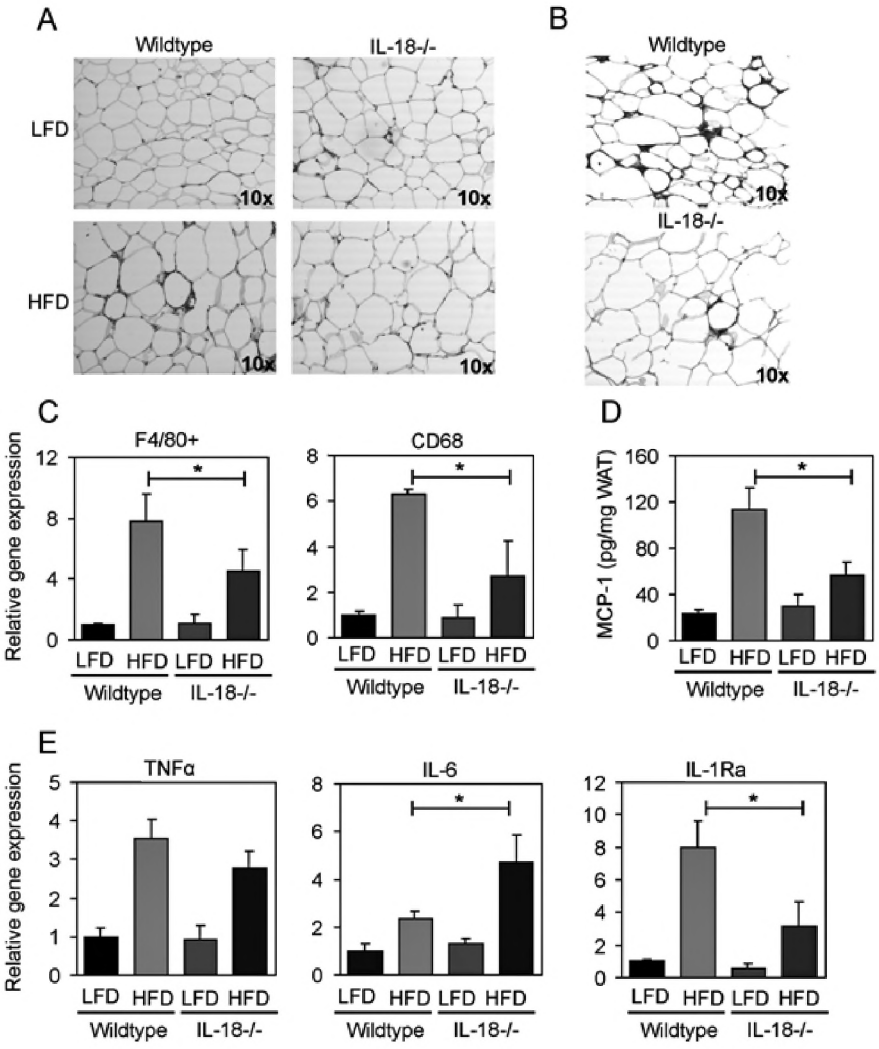

Figure 5 **White adipose tissue specific effects of IL-18 in diet-induced obesity**. A. Representative HE staining of white adipose tissue of wild-type and IL-18-/- mice fed a HFD or a LFD for 16 weeks. B. Representative immunohistochemical staining with anti-F4/80+ of white adipose tissue of a wild-type and IL-18-/- mouse fed a HFD for 16 weeks. C. Gene expression levels of the macrophage markers F4/80+ and CD68 in white adipose tissue of wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group). D. Adipose tissue levels of MCP-1 wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group). E Gene expression levels of the pro-inflammatory cytokines TNF-α and IL-6 and the anti-inflammatory cytokine IL-1Ra in white adipose tissue of wild-type and IL-18-/- animals fed a HFD or a LFD for 16 weeks (n=10 per group).Data are presented as means ± SEM. Asterisks represents statistically significant differences between both genotypes within the same dietary intervention group. * = P-value<0.05.

protective role of IL-18 in the development of high fat diet induced insulin resistance. Several pro-inflammatory cytokines, including IL-1β, have been shown to negatively interfere with insulin signaling pathways. In vitro, cytokines can inhibit phosphorylation of insulin receptor substrates ultimately leading to insulin resistance and the development of Type 2 diabetes mellitus (22;23). In vivo, plasma concentrations of cytokines are often positively correlating with the severity of obesity or presence of insulin resistance (24). Recently published studies have suggested that members of the IL-1 family of cytokines including IL-18 contribute to the development of insulin resistance (25;26). Our data revealed high steady-state gene expression and protein levels of IL-18 in liver and white adipose tissue. In line with its high basal expression levels, absence of IL-18 in mice fed a HFD led to disturbances in energy homeostasis and reduced insulin sensitivity exemplified by a lower plasma adiponectin concentration and increased TG levels. Additionally, HFD feeding enhanced plasma ALT liver enzymes in the absence of IL-18. Whereas acute IL-18 treatment has been reported to induce liver failure and injury (27), other studies have shown a protective function of IL-18 (28). Our data provides additional indications for a protective role of IL-18 in liver during chronic overfeeding.

In contrast to other members of the IL-1 family of cytokines with low or absent basal expression levels, the constitutive expression is suggestive of a more endocrine function of IL-18. Hypothetically, IL-18 plays an important role in controlling insulin signaling pathways supported by the impaired insulin sensitivity observed in IL-18-/- mice and improvement of insulin sensitivity after treatment with recombinant IL-18. The precise mechanisms or signaling pathways by which IL-18 may control insulin signaling are currently unknown and deserve further study.

Paradoxically, observations in humans have shown that IL-18 concentrations are increased in individuals with type 2 diabetes mellitus and obesity and infusion of glucose leads to an acute increase in serum IL-18 concentrations (15;29). Only recently, it has been reported that in vitro cultured adipocytes treated with recombinant IL-18 have lower adiponectin secretion as compared to untreated cells (30). These results imply that IL-18 induces the development of insulin resistance. However, it has also been shown that *in-vitro* treatment of adipocytes with IL-18 enhances insulin-dependent glucose uptake (31). It can be envisioned that during the development of obesity a reduced sensitivity towards IL-18 is developed similar to insulin or leptin resistance. Subsequently, IL-18 resistance is compensated by a higher production of IL-18 to overcome the resistance. Additionally, the development of obesity induces down regulation of IL-18 receptor complexes (9;16) or may result in up regulation of IL-18bp thereby leading to blockage of IL-18 signaling pathways and resulting in IL-18 resistance. Although increased levels of IL-18 have been frequently linked to the development of obesity and insulin resistance, mechanistic studies to decipher the regulation of IL-18 are scarce. Our data suggest that IL-18 protects against the development of obesity-induced insulin resistance and contributes to effective hepatic

insulin signaling. These effects may partially be effectuated by regulating adiponectin secretion, since HFD-fed IL-18-/- animals suffer from lower circulating concentrations of this insulin-sensitizing protein (32).
Surprisingly, absence of IL-18 prevented the HFD-induced influx of macrophages into the adipose tissue as both immunohistochemical analysis and qPCR revealed a reduced number of macrophages present in adipose tissue of IL-18-/- animals fed the HFD as compared to wild-type mice. IL-18 has been shown to regulate MCP-1 gene expression and protein levels (33) and we observed lower adipose tissue concentrations of MCP-1 in IL-18-/- animals. Inasmuch macrophage influx into the inflammatory adipose tissue has been clearly linked to the development of systemic insulin resistance, the absence of IL-18 appears to overrule these effects in determining systemic insulin sensitivity. In contrast, the absence of IL-18 led to enhanced expression of IL-6 and reduced expression of IL-1Ra in adipose tissue. Therefore, IL-18 may be involved in limiting pro-inflammatory activity in adipose tissue during chronic overfeeding and thereby reduce the development of systemic insulin resistance.

Besides its role in insulin signaling, IL-18 has been shown to control food intake that may involve controlling hypothalamic neuropeptides levels, which regulates feeding behaviour. Although IL18-/- animals overate the LFD, HFD-fed IL-18-/- mice did not display significant differences in food intake compared to wild-type mice. This suggests that IL-18 controls macronutrient-specific food intake other than that of fat. One potential candidate neuropeptide to be involved in IL-18-controlled feeding behaviour is orexin/hypocretin, as hypothalamic gene expression levels were significantly lower in LFD fed IL-18-/- animals. Although acute orexin treatment propagates appetite, chronic activation of orexin signaling pathways has been shown to promote energy expenditure and reducing consumption (34). By regulating hypothalamic levels of orexin, IL-18 may reduce food intake. Overall, the relatively small changes observed in hypothalamic neuropeptide levels of NPY and AGRP and unchanged levels of POMC and CART between wild-type and IL-18-/- animals do not support a substantial role of IL-18 in controlling neuropeptide gene expression levels.
In contrast to previous reports, the HFD intervention did not lead to changes in bodyweight gain in IL-18-/- animals compared to wild-type mice (10). The age of the animals at the start of our study (3 months) or differences in diet composition may explain the lack of bodyweight differences between both genotypes.

In conclusion, our results suggest an important and direct role of IL-18 in controlling insulin sensitivity independently of the development of obesity, liver steatosis and macrophage influx into the adipose tissue. Although our results concerning food intake and bodyweight development partly differ from earlier studies, our data demonstrates that IL-18 protects against the development of insulin resistance due to HFD feeding.

Acknowledgements

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Chapter 5

The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity

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Abstract

Obesity-induced inflammation originating from expanding adipose tissue interferes with insulin sensitivity. Important metabolic effects have been recently attributed to IL-1β and IL-18, two members of the IL-1 family of cytokines. Processing of IL-1β and IL-18 requires cleavage by caspase-1, a cysteine protease regulated by a protein complex called the inflammasome. We demonstrate that the inflammasome/caspase-1 governs adipocyte differentiation and insulin sensitivity. Caspase-1 is upregulated during adipocyte differentiation and directs adipocytes towards a more insulin-resistant phenotype. Treatment of differentiating adipocytes with recombinant IL-1β and IL-18, or blocking their effects by inhibitors, reveals that the effects of caspase-1 on adipocyte differentiation are largely conveyed by IL-1β. Caspase-1 and IL-1β activity in adipose tissue is increased both in diet- and genetically-induced obese animal models. Conversely, mice deficient in caspase-1 are more insulin sensitive as compared to wild-type animals. In addition, differentiation of pre-adipocytes isolated from caspase-1-/- or NLRP3-/- mice resulted in more metabolically active fat cells. In vivo, treatment of obese mice with a caspase-1 inhibitor significantly increases their insulin sensitivity. Indirect calorimetry analysis revealed higher fat oxidation rates in caspase-1-/- animals. In conclusion, the inflammasome is an important regulator of adipocyte function and insulin sensitivity, and caspase-1 inhibition may represent a novel therapeutic target in clinical conditions associated with obesity and insulin resistance.

Introduction

The prevalence of obesity, an important risk factor for type 2 diabetes, hypertension and hyperlipidemia, has reached epidemic proportions worldwide (1). Initially viewed as a tissue that merely stores energy, recently it has become evident that adipose tissue also releases hormone-like mediators named adipokines, and exerts effects reminiscent of an endocrine organ (2). During the development of obesity, the morphology and functional properties of adipose tissue change dramatically. In addition to adipocyte hypertrophy due to storage of increasing amounts of lipids, adipose tissue turns into an inflamed tissue characterized by macrophage infiltration and altered secretion of adipokines (3;4). Whereas the secretion of pro-inflammatory cytokines is enhanced, the production of insulin-sensitizing adipokines such as adiponectin is reduced. To date, many pro-inflammatory cytokines have been linked to the development of insulin resistance and type 2 diabetes including TNFα and IL-6 (3;5). More recently, interest has grown into the role of the IL-1 family of cytokines and its prominent members IL-1β and IL-18. IL-1β is a pro-inflammatory cytokine with a role in the pathogenesis of type 1 diabetes through its toxic effects on beta-cells of the pancreas (6). It has been shown that treatment of type 2 diabetic patients with IL-1Ra, an antagonist of IL-1 signaling, improves glycemic control and beta-cell function (7). Recent work has also revealed an important role of IL-18 in modulation of energy balance and insulin sensitivity (8).
In order to be activated, IL-1β and IL-18 have to be processed from inactive precursors by an intracellular cysteine protease called caspase-1 (9;10). Activation of caspase-1 itself takes place by conformational changes in a protein platform called the inflammasome, consisting of caspase-1 and proteins of the NACHT-LRR (NLR) family including NLRP3 and ASC, leading to the release of the active enzyme (11). The inflammasome is activated in response to so-called danger-associated molecular patterns, which can be elicited either by microbial components (e.g. peptidoglycan) or by endogenous danger signals (e.g. uric acid, ATP) (9). Because of the metabolic effects described for IL-1β and IL-18, and due to the crucial role of caspase-1 for the activation of these cytokines, we hypothesized that inflammasome-dependent activation of caspase-1 plays an important role in the metabolic function of the adipose tissue. This hypothesis was tested using a number of *in vitro* and *in vivo* approaches.

Methods

Animal studies

IL-1β−/− mice were a gift from J.Mudgett, Merck Research Laboratories, Rahway, USA. Caspase-1-/- mice were a kind gift from Prof. Dinarello, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262, USA.

NLRP3-/- mice were generated as described previously (12). All mice were back-crossed ten generations to C57Bl/6 mice, and age-matched wild-type C57Bl/6J mice were used as controls throughout the different experiments. Db/Db and Ob/Ob mice in a C57Bl/6 background were obtained from the Jackson Laboratories.

Diet intervention

Wild-type C57Bl/6J male mice received a low fat diet (LFD) or a high fat diet (HFD) for 16 weeks, providing 10 or 45% energy percent in the form of triglycerides (D12450B or D12451, Research Diets, New Brunswick, USA). In the last week of dietary intervention, some animals were injected intraperitoneally with clodronate liposomes. The liposomes were prepared as described previously (13) using phosphatidylcholine (Lipoid GmbH, Ludwigshafen,Germany) and cholesterol (Sigma Chem.Co. USA). Cl2MDP (or clodronate) was a gift of Roche Diagnostics GmbH, Mannheim, Germany. Caspase-1-/- and wild-type mice were fed the similar LFD or HFD during 10 weeks. Insulin-tolerance tests were performed as previously described (14). Briefly, animals were fasted for 4 hours and insulin was given intraperitoneally at a dose of 0.75 U/kg bodyweight. Blood samples were taken before and 30, 60, 90 or 120 minutes after insulin administration. Plasma levels of insulin and adiponectin secretion in cell culture medium were measured using ELISA (LINCO Research Inc., St. Charles, MO, USA).

Euglycemic hyperinsulinemic clamp analysis

Euglycemic hyperinsulinemic clamps were performed as described earlier (15). Briefly, after an overnight fast, animals were anesthetized with 6.25mg/kg acepromazine (Sanofi Santé Nutrition Animale, Libourne Cedex, France), 6.25mg/kg midazolam (Roche, Mijdrecht, The Netherlands) and 312.5µg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands) and an infusion needle was placed in the tail vein. A bolus of insulin (3mU) was given, and a hyperinsulinemic euglycemic clamp was started with a continuous infusion of insulin (5mU/h) and a variable infusion of 12.5% D-glucose (in PBS) to maintain blood glucose level at euglycemic levels. Blood samples were taken every 5 to 10 minutes from the tip of the tail to monitor plasma glucose levels (AccuCheck). 70, 80 and 90 minutes after the start of the clamp, blood samples (70 µl) were taken for determination of plasma glucose, insulin and FFA concentrations. Plasma glucose, insulin and FFA levels were determined using commercially available kits (Instruchemie, crystal chem. Inc. and Wako Pure Chemical Industries respectively).

Indirect calorimetry

Animals were housed in a controlled environment (23°C, 55% humidity) under a 12h light-dark cycle (07:00-19:00). Food and tap water was available ad libitum during the whole experiment. Mice were fed normal laboratory chow (RM3, Special Diet

Services, UK) throughout the experiment. Indirect calorimetry was performed for a period of 60 hours (Comprehensive Laboratory Animal Monitoring System, Columbus Instruments, Columbus Ohio, US). An acclimatization period of 24 hours prior to the start of the experiment was included. Food intake, oxygen consumption (VO2) and carbon dioxide production rates (VCO2) were measured at intervals of 7 minutes. Respiratory exchange ratio (RER) was calculated as the ratio between VCO2 and VO2. Absolute oxidation rates for fat and carbohydrate were calculated according to Peronnet and Massicotte (16). Fecal output was determined at the end of the experiment. Fat content of the faeces was analyzed using the acid steatocrit method as described previously (17).
All the animal studies were approved by the animal experimentation committee of Wageningen University, Leiden University Medical Center, Radboud University Nijmegen Medical Centre, the Netherlands, the University of Illinois at Chicago, and St Jude Children's Research Hospital, Memphis, Tennessee, United States of America.

IL-1β and IL-18 measurements

IL-1β concentrations in white adipose tissue or cell culture medium were determined using a specific radioimmunoassay as previously described (18). IL-18 concentrations in white adipose tissue were measured using a commercially available ELISA (Invitrogen). Bio-active IL-1 was measured using the murine thymoma cell line EL4/NOB 1 that produces IL-2 in response to bioactive IL-1 (19). IL-2 levels were subsequently measured by ELISA (R&D).

Cell culture

Mouse 3T3-L1 cells and human SGBS cells were cultured and differentiated towards adipocytes as described previously (20;21). Oil red O staining of cells was performed using a standard protocol. Oil red O staining was quantified by using total photographic images of the staining which were divided in multiple fields and the number of positive cells per field were counted. 2 hours before insulin treatment of cells to assess insulin sensitivity, cells were cultured in serum-free medium.

Caspase-1 activity assay

Caspase-1 activity was measured in isolated adipocytes and total white adipose tissue using a fluorometric assay (Biovision, California, USA) following manufacturer's instructions with slight modifications. Briefly, total adipose tissue or isolated adipocytes were homogenized in lysis buffer and caspase-1 activity was measured by the addition of the caspase-1 specific peptide YVAD-AFC. Cleavage of the substrate by caspase-1 releases AFC that can subsequently be quantified using a fluorimeter. By comparing the fluorescence of AFC from an untreated sample with a sample treated with the specific caspase-1 inhibitor Pralnacasan, caspase-1 activity can be determined.

Isolation and culturing of primary adipocytes and white adipose tissue explants

Freshly isolated mouse epididymal adipose tissue was used for the isolation of adipocytes and stromal vascular cells. Minced adipose tissue was digested using collagenase (Sigma-Aldrich) at a concentration of 5mg/ml dissolved in Dulbecco's Modified Eagle's Medium (DMEM). Tissues were incubated for 45 minutes at 37°C and were subsequently filtered through a 250µM nylon mesh filter. After centrifugation, the floating cells were collected as adipocytes and the pelleted cells as stromal vascular cells. Stromal vascular cells were cultured and differentiated towards adipocytes using standard protocols. A similar protocol was applied for the isolation of human adipocytes and stromal vascular cells. For culturing of white adipose tissue explants, freshly isolated fat was minced and directly brought into culture using Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. Human adipose tissue was obtained from patients undergoing reconstructive abdominal surgery after written informed consent.

Histology

Morphometry of individual fat cells was assessed using digital image analysis. Microscopic images were digitized in 24 bit RGB (specimen level pixel size 1.28x1.28um^2). Recognition of fat cells was initially performed by applying a region growing algorithm on manually indicated seed points, and minimum Feret diameter were calculated. Differences between groups were studied using one-way univariate Anova. Tukey HSD post hoc testing was applied for multiple comparison testing.

RNA and DNA isolation and qPCR analysis

RNA from animal tissues was isolated using Trizol Reagent (Invitrogen) following manufacturer's instructions. RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Real-Time PCR was done with a Power Sybr Green PCR master mix (Applied Biosystems) using a 7300 Real-Time PCR System (Applied Biosystems). Melt curve analysis was included to assure a single PCR product was formed. Values were corrected using the housekeeping gene 36B4 or beta2-microglobulin (β2M). DNA was isolated from adipose tissue and mitochondrial DNA was quantified using primers to detect mitochondrial D-loop. Results were corrected for the total amount of DNA within samples using primer sequences to detect LPL. Primer sequences are available upon request.

Immunoblot analysis

Immunoblotting was carried out using an ECL system (Amersham Biosciences, Diegem, Belgium). Equal amounts of cell lysates as determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories BV) were resolved by SDS/PAGE on a 12% polyacrylamide gel. Proteins were transferred using the iBLOT system (Invitrogen)

following manufacturer's instructions. The pAKT and AKT antibody (R&D Systems), caspase-1 antibody (Santa Cruz) and actin antibody (Sigma-Aldrich) were used at a dilution of 1:1000 and the membranes were incubated overnight at 4˚C. The secondary antibodies were used at a dilution of 1:5000. All incubations were performed in 1x Tris-buffered saline, pH 7.5, with 0.1% Tween 20 and 5% dry milk. In the final washings, dry milk was removed from the solution.

Statistical analysis

Statistical significant differences were calculated using a Student's T-test. The cut-off for statistical significance was set at a *P*-value of 0.05 or below.

Results

Expression and function of caspase-1 in adipocytes

To explore the role of the caspase-1 in adipocyte function, we first assessed the expression of caspase-1 during *in vitro* adipogenesis. Interestingly, differentiation of mouse 3T3-L1, human SGBS (Simpson-Golabi-Behmel Syndrome) and human primary adipocytes was associated with a significant increase in expression of caspase-1 in parallel with the adipogenic transcription factor PPARγ (Figure 1A). IL-1β gene expression levels during adipocyte differentiation displayed a more variable pattern (Figure 1B).

Caspase-1 protein levels were significantly higher in differentiated cells (Figure 1B) and caspase-1 activity levels were increased in fully differentiated adipocytes vs. preadipocytes (Figure 1C). In order to assess whether caspase-1 influences adipocyte differentiation, we blocked caspase-1 with the caspase-1 inhibitor Pralnacasan (22). This inhibitor more effectively reduced caspase-1 activity compared to the frequently used caspase-1 inhibitor Yvad as determined by an *in vitro* caspase-1 activity assay (supplement Figure 1A). Caspase-1 blockade improved expression levels of adipogenic marker genes including PPARγ, adiponectin and Glut4 gene expression (Figure 1D). Importantly, caspase-1 blockage in human adipose tissue using Pralnacasan reduced the secretion of IL-1β (Supplement Figure 1B). In addition to caspase-1 inhibition using an inhibitor, caspase-1 function was silenced using siRNA. Treatment of cells with siRNA targeted to caspase-1, led to a significant reduction in caspase-1 gene expression (relative gene expression levels: 1.00 ± 0.04 vs. 0.16 ± 0.01). In addition to mRNA levels, caspase-1 protein levels were decreased in cells transected with siRNA targeted against caspase-1 (Supplemental Figure 1G). Basal secretion levels of bio-active IL-1, as measured by using the murine thymoma cell line EL4/NOB 1 that produces IL-2 in response to bioactive IL-1, were unchanged in cells depleted of caspase-1. Probably, levels of IL-1 produced by the cells under normal conditions are relative low and the secreted IL-1 may directly bind to the cell membrane.

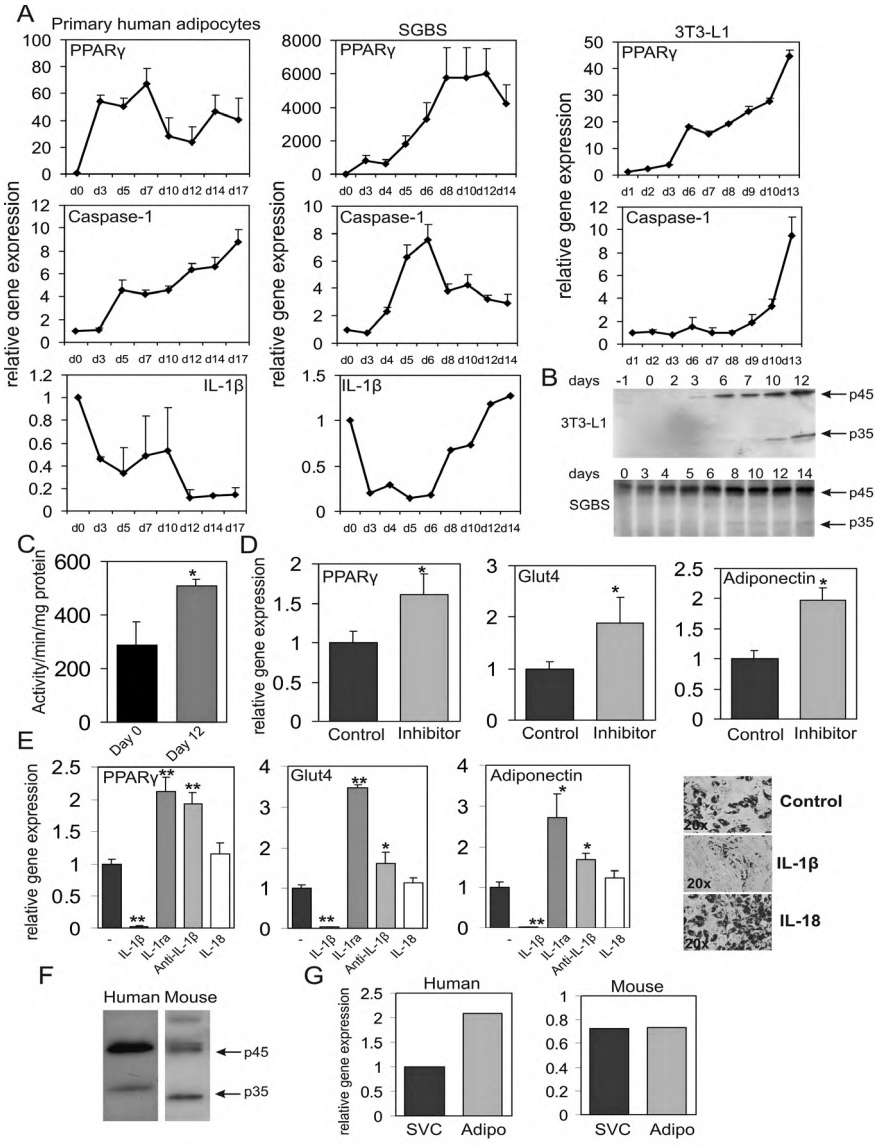

Figure 1 **Caspase-1 is activated during adipogenesis *in-vitro* and is present in adipocytes of mouse and human white adipose tissue *in-vivo*.** A, quantitative PCR analysis of differentiating human or mouse adipocytes. Representative results are shown of n=3 experiments. B, Western blot analysis of caspase-1 protein levels in differentiating mouse 3T3-L1 adipocytes and human SGBS adipocytes. Protein marker is given in kDalton. C, caspase-1 activity in pre-adipocytes (Pre) and adipocytes at day 8 of differentiation (Mature). D, quantitative PCR analysis of SGBS adipocytes differentiated for 12 days in the presence of the specific caspase-1 inhibitor Pralnacasan at 100μM. E, quantitative PCR analysis and representative oil red O staining of SGBS adipocytes differentiated for 7 days in the presence of recombinant IL-1β (10ng/ml), IL-1ra (5μg/ml), Anti-IL-1β antibody (5μg/ml) or IL-18 (25ng/ml). F, Western blot analysis of caspase-1 in total human and mouse white adipose tissue. Protein marker is given in kDalton.

G, caspase-1 expression in the stromal vascular cells and mature adipocytes fractionated from total white adipose tissue. Data are presented as mean ± SEM. Asteriks depict statistically significant differences between control and experimental groups. * P-value< 0.05, ** P-value< 0.005.

Gene expression analysis of PPARγ and adiponectin revealed that knockdown of caspase-1 led to a significant increase in expression of 4.01 +/-1.12 fold of PPARγ and 1.88 +/-0.19 fold of adiponectin compared to mock treated cells (data not shown). Silencing of caspase-1 in SGBS adipocytes improved insulin sensitivity as determined by pAKT/AKT ratios after short term insulin treatment (mock vs. siRNA Caspase-1: 0.95 ± 0.14 vs. 2.10 ± 0.16. P-value < 0.005). Finally, treatment of caspase-1-depleted cells with recombinant IL-1β led to a reduction in pAKT levels (Supplemental Figure 1H).

To examine whether the effects of caspase-1 may be mediated by processing of IL-1β or IL-18, we studied the effects of these cytokines on adipocyte differentiation. Whereas recombinant IL-1β blocked adipogenesis, as assessed by oil Red O staining, recombinant IL-18 had no affect (Figure 1E). Importantly, treatment of differentiating adipocytes with recombinant IL-1 receptor antagonist to block endogenous IL-1 signaling mirrored the effects of caspase-1 inhibition (Figure 1E). Moreover, specific blockage of IL-1β-signaling pathways in differentiating SGBS adipocytes using an anti-IL-1β antibody also resulted in an improvement of adipogenesis (Figure 1E). Thus, effects of caspase-1 on adipogenesis are likely conveyed via endogenous IL-1β. In order to confirm that caspase-1 has a functional role in vivo, caspase-1 protein levels were assessed in human and mouse white adipose tissue. Both human and mouse white adipose tissue displayed total (45kDA) and activated caspase-1 isoforms (34kDA) (Figure 1F). Fractioning of total human and mouse white adipose tissue into mature adipocytes and stromal vascular cells revealed that caspase-1 is expressed in both cell populations (Figure 1G). Taken together, our data show that caspase-1 is up regulated during in-vitro adipocyte differentiation and is present in mouse and human white adipose tissue in vivo.

Caspase-1 function in experimental models of obesity

To determine caspase-1 function in adipose tissue during diet-induced obesity, C57Bl/6 mice were fed a high fat diet (HFD) for 16 weeks. HFD feeding caused obesity as illustrated by increased bodyweight (Figure 2A) and epididymal adipose tissue mass (Figure 2B). Importantly, after prolonged HFD, adipose tissue expression of caspase-1 was markedly increased (Figure 2C). A similar increase in caspase-1 mRNA and protein was observed in obese db/db mice and Ob/Ob mice (Figure 2D). Protein levels of the active isoform of caspase-1 (34kDA) were increased in white adipose tissue of obese animals (Figure 2E). In line with elevated gene expression and protein levels, caspase-1 activity was also increased in total white adipose tissue of HFD and Ob/Ob animals compared to lean mice (Figure 2F). The increased caspase-1 activity

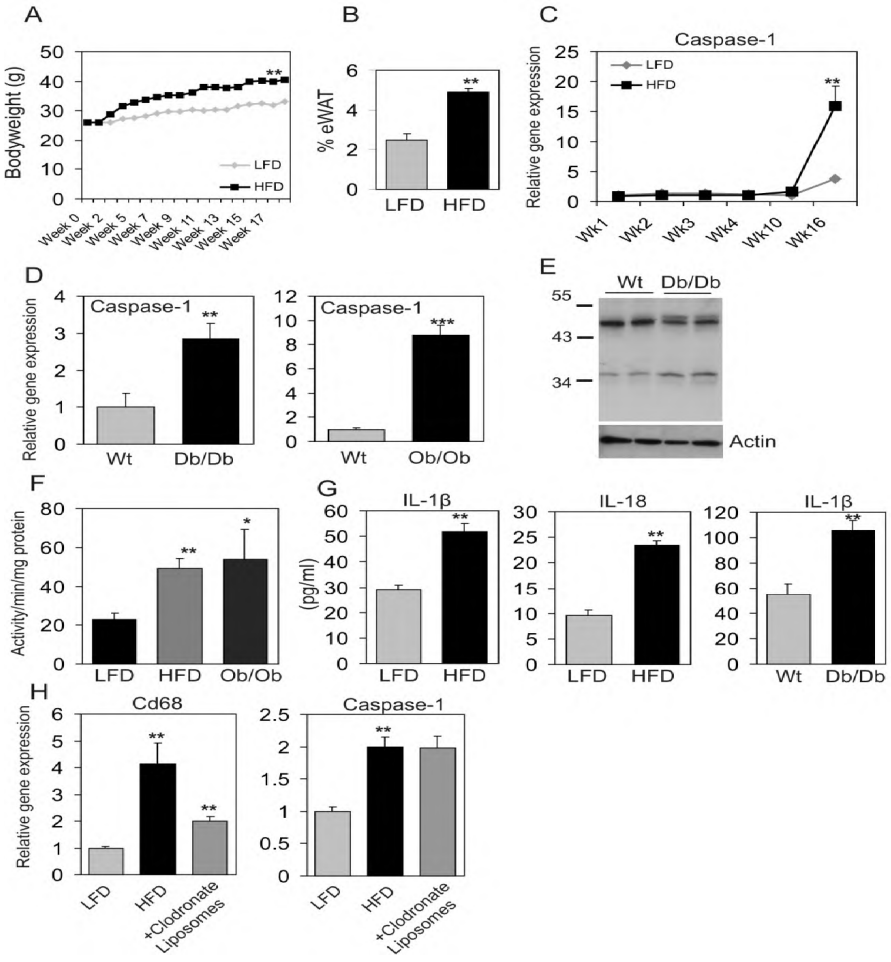

Figure 2 **Caspase-1 is activated in adipocytes of diet-and genetically induced obese animals**. A, Bodyweight development in male wild-type C57/bl6 animals (2 months of age at start of intervention) fed either a low or high fat diet (n=10 per group). B, % of epididymal white adipose tissue after 17 weeks of high fat diet feeding. C, quantitative PCR analysis of caspase-1 gene expression in white adipose tissue of C57/Bl6 animals during dietary intervention (n=6 per group). D, white adipose tissue of wild-type, Db/Db and Ob/Ob animals (4 months of age) is analyzed for caspase-1 gene expression levels using quantitative PCR analysis (n=5 per group). E, Western blot analysis of caspase-1 in white adipose tissue of wild-type and Db/Db animals. Actin levels are shown as loading control. F, caspase-1 activity levels measured in total white adipose tissue of LFD or HFD fed animals and Ob/Ob mice. G,IL-1β and IL-18 concentrations (pg/ml) measured in total white adipose tissue of wild-type animals fed a LFD or HFD and IL-1β concentrations (pg/ml) present in white adipose tissue of wild-type and Db/Db mice (n=5 per group). H, Gene expression levels of CD68 and caspase-1 in white adipose tissue of wild-type animals (2 months of age at start of intervention) fed a low fat diet or a high fat diet for 16 weeks and a high fat diet followed by intraperitoneal injection with clodronate liposomes. Data are presented as mean ± sem. Asterisks depict statistically significant difference between control and experimental groups. * P-value<0.05, ** P-value<0.005, *** P-value<0.001.

was accompanied by significantly elevated IL-1β and IL-18 protein levels in adipose tissue of diet- and genetically-induced obese mice (Figure 2G). Although IL-1β and IL-18 protein levels were elevated, we were unable to detect any significant changes in gene expression of IL-1β and IL-18 in total adipose tissue of obese vs. lean animals.

Previous studies have shown that obesity and adipocyte hypertrophy result in an influx of macrophages into adipose tissue (4;23), and we assessed whether the increased expression of caspase-1 in adipose tissue was due to an increase in the infiltrating macrophages or the adipocytes themselves. Partial depletion of macrophages from adipose tissue of animals fed a HFD decreased the expression of the macrophage marker CD68, yet did not alter the expression of caspase-1 (Figure 2H), suggesting that caspase-1 effects on adipose tissue are not only exerted through infiltrating macrophages. Together, these data show that both diet-and genetically induced obesity result in activation of caspase-1 and increased levels of IL-1β and IL-18 in adipose tissue.

Caspase-1 modulates adipocyte function

To assess in more detail the role of caspase-1 in adipocyte function, pre-adipocytes from caspase-1-deficient mice and wild-type mice were differentiated towards adipocytes using a standard adipocyte differentiation protocol. In the absence of caspase-1, adipogenesis was enhanced, as illustrated by oil red O staining (Figure 3A) and increased gene expression levels of GLUT-4, adiponectin and PPARγ (Figure 3B). Importantly, secretion of adiponectin, a key regulator of insulin sensitivity (24), was significantly elevated in caspase-1 deficient adipocytes (Figure 3C). Additionally, insulin signaling in caspase-1-deficient adipocytes was ameliorated, as visualized by an increased phosphorylation of Akt (Figure 3D) (Relative pAKT/AKT ratio: wild-type 10nm insulin vs. Caspase-1-/- 10nm insulin: 1 ± 0.53 vs. 3.09 ± 0.12, P-value<0.05 ; wild-type 100nm insulin vs. Caspase-1-/- 100nm insulin: 4.13 ± 1.33 vs. 8.85 ± 1.8, P-value<0.05). Importantly, in mature human adipocytes made insulin resistant by overnight treatment with 200nM of insulin, caspase-1 inhibition increased insulin action as determined by pAKT levels after 20 minutes of insulin treatment (50nM) (Figure 3E) (Relative pAKT/AKT ratio: Control vs. inhibitor: 1 ± 0.24 vs. 2.41 ± 0.15, P-value<0.01).

Because NLRP3 is an important component of the inflammasome involved in the activation of caspase-1 (9), we investigated whether absence of NLRP3 mimics the effects seen in caspase-1 deficient animals. Adipocyte differentiation of pre-adipocytes isolated from NLRP3-deficient animals was similarly enhanced compared to caspase-1 deficient cells (Figure 3F and G). Finally, we tested insulin sensitivity of total white adipose in wild-type, caspase-1-/- and NLRP3-/- animals. As shown in figure 3H, the absence of caspase-1 and NLRP3 in white adipose tissue resulted in an increase in adipose tissue insulin sensitivity as determined by phosphorylation of

AKT (Relative pAKT/AKT ratio: wild-type vs. Caspase-1-/- vs. NALP3-/- , 10nM insulin: 4.06 ± 0.54 vs. 11.20 ± 1.66 vs. 12.11 ± 2.19, *P*-value<0.05, 100nm insulin: 5.56 ± 0.43 vs. 14.47 ± 2.88 vs. 29.23 ± 7.28, *P*-value<0.05) . In line with the rise in insulin sensitivity, IL-1β production of adipose tissue isolated from caspase-1-/- and NLRP3-/- animals was significantly reduced as compared to white adipose tissue from wild-type mice (Figure 3I). These data establish an important function of caspase-1 and NLRP3 in adipocyte formation and insulin sensitivity.

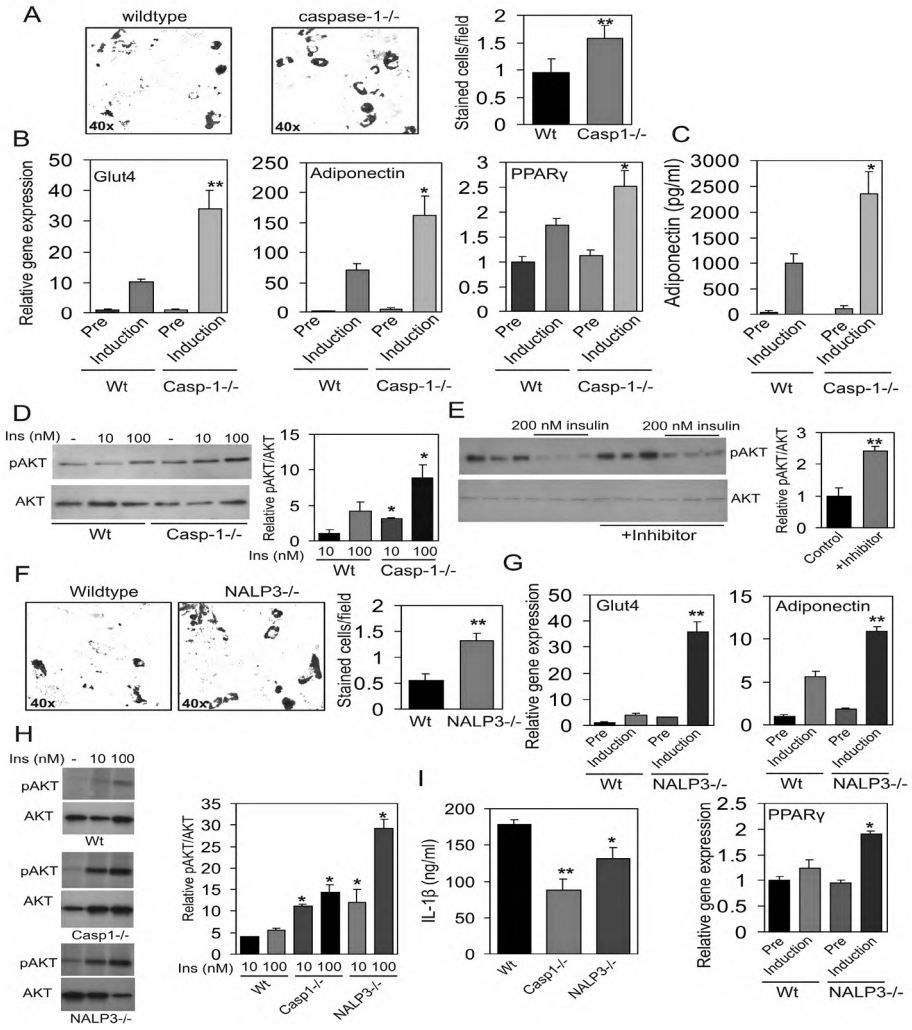

Figure 3 **Absence of caspase-1 and NLRP3 improves adipogenesis and insulin sensitivity in differentiated adipocytes**. A, Representative Oil red O staining pictures and quantification of wild-type and caspase-1-deficient pre-adipocytes differentiated towards adipocytes for 7 days. B, GLUT4, Adiponectin and PPARγ gene expression levels as determined by quantitative real-time PCR in pre-adipocytes (Pre)

or differentiated adipocytes (Induction) from wild-type or caspase-1 deficient animals. C, Adiponectin concentrations (pg/ml) measured in medium of pre-adipocytes (Pre) or adipocytes (Induction) differentiated for 7 days from wild-type or caspase-1 deficient animals. D, Western blot analysis of phosphorylated AKT and total AKT levels after insulin treatment for 20 minutes in adipocytes differentiated for 7 days. E, Western blot analysis of phosphorylated AKT and total AKT levels after 20 minutes of insulin treatment in mature SGBS adipocytes pre-treated overnight with 200nM of insulin and/or the caspase-1 inhibitor pralnacasan (100μM). F, Representative Oil red O staining pictures and quantification of wild-type and NLRP3-deficient pre-adipocytes differentiated towards adipocytes for 7 days. G, GLUT4, Adiponectin and PPARγ gene expression levels in pre-adipocytes (Pre) or differentiated adipocytes (Induction) from wild-type or NALP3 deficient animals. H, Western blot analysis of phosphorylated AKT and total AKT levels in white adipose tissue explants from wild-type, caspase-1-/- and NLRP3-/- animals (2 months of age) after 20 minutes of insulin treatment. I, IL-1β production of adipose tissue explants from wildtype, caspase-1-/- and NLRP3-/- animals after 24 hours of culturing. Data are presented as mean ± sem. Asterisks depict statistically significant differences between control and experimental groups. * *P*-value< 0.05, *** *P*-value< 0.005.

Absence of caspase-1 improves insulin sensitivity in vivo

To unveil the potential role of caspase-1 in vivo, adipose tissue function and morphology was analyzed in caspase-1-deficient animals. The absence of caspase-1 led to a profound change in adipose tissue morphology (Figure 4A) with markedly smaller adipocytes (average adipocyte size: wild-type mice 966,8μM^2, Caspase-1-/- mice 629,16μM^2, *P*-value < 0.001) (Figure 4A). In addition, although total bodyweight was not different between both genotypes (Figure 4B), DEXA scan analysis of caspase-1-/- animals revealed a profound reduction in total fat mass (Figure 4B). On the other hand, bone mineral content was significantly elevated in caspase-1-/- animals compared to wildtype mice (Figure 4B).

In line with the beneficial change in adipose tissue morphology, gene expression analysis revealed elevated levels of PPARγ, adiponectin and GLUT4, consistent with increased insulin sensitivity and improved glucose uptake in adipose tissue (Figure 4C). In addition, adipose PGC1α and PGC1β gene expression levels were highly elevated in caspase-1 deficient animals (Figure 4C), implying an increase in mitochondrial activity and energy expenditure (25). Indeed, quantification of mitochondrial DNA content in WAT of wildtype and caspase-1-/- animals revealed a robust increase in mtDNA of white adipose tissue in the absence of caspase-1 (Figure 4D).

Finally, our in vitro observations showing enhanced insulin action in caspase-1 deficient adipocytes were accompanied by an improvement in total insulin sensitivity in caspase-1 deficient animals, as illustrated by a decrease in basal plasma insulin levels and lower plasma glucose levels during insulin tolerance tests (Figure 4E and F). Assessment of whole body insulin resistance by euglycemic hyperinsulinemic clamp analysis revealed a significant improvement of insulin sensitivity in caspase-1-/- animals as compared to wild-type mice (Figure 4G, Supplement Figure 1E, Supplementary table 1).

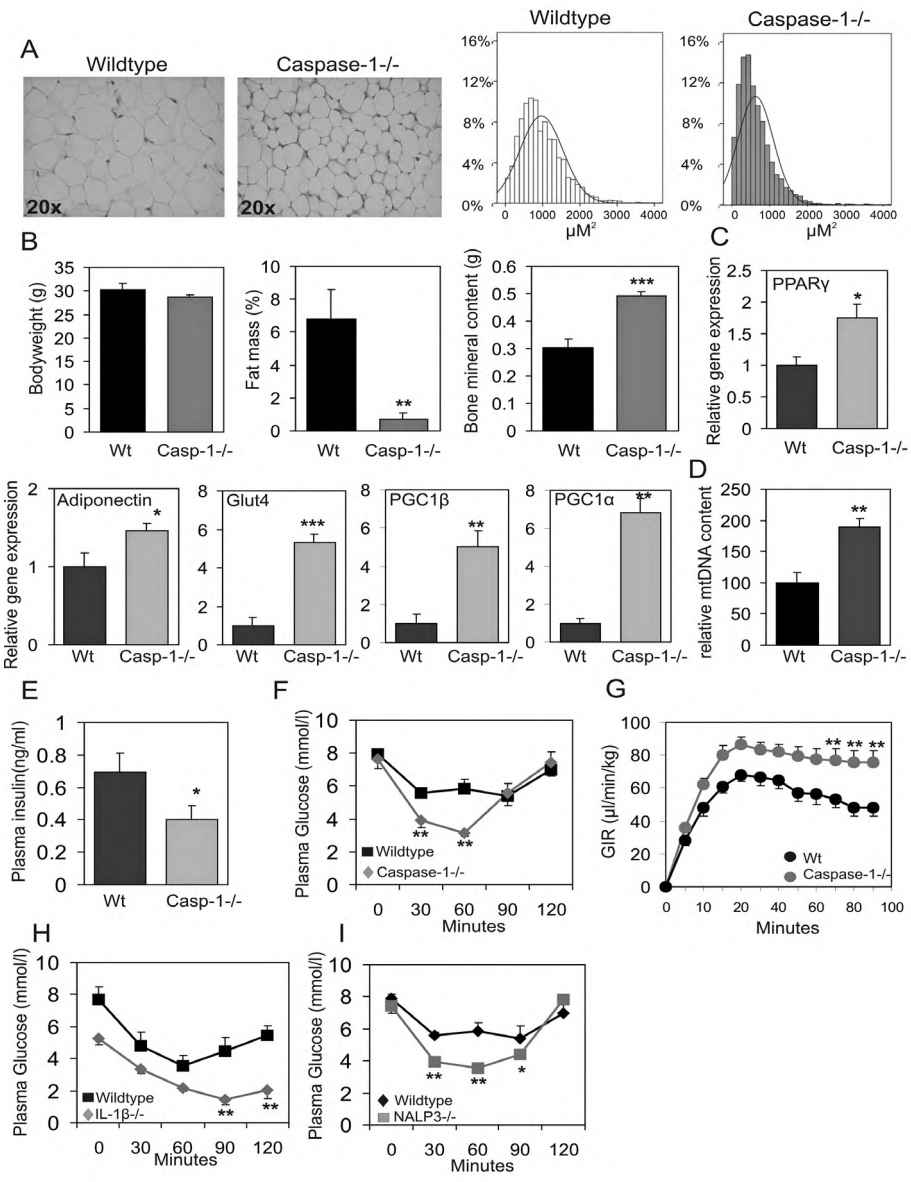

Figure 4 **Caspase-1 contributes to adipose tissue formation and function *in vivo***. A, Representative HE-staining of white adipose tissue and quantification of adipocyte size. B, Total bodyweight, percentage of fat mass and bone mineral content of wild-type and capase-1-/- mice (6 months of age). C, gene expression analysis of total white adipose of wildtype and caspase-1 deficient mice using real-time quantitative PCR techniques. D, mtDNA content of total white adipose tissue from wildtype and caspase-1-/- animals. E, Plasma insulin levels in non-fasted wild-type and caspase-1 deficient animals (n=9 per group). F, Insulin tolerance test in wild-type and caspase-1-deficient animals (n=9 per group). G, Assessment of whole body insulin resistance by euglycemic hyperinsulinemic clamp analysis in wild-type (n=17) and caspase-1-/- (n=18) animals at 1 year of age.

Glucose infusion rates (GIR) during the euglycemic hyperinsulinemic clamp analysis are shown. H, Insulin tolerance test in wild-type and IL-1β-deficient animals (n=5 per group). I, Insulin tolerance test in wildtype and NALP3-deficient animals (n=5 per group). Data are presented as mean ± sem. Asterisks depict statistically significant differences between control and experimental groups.* P-value< 0.05, ** P-value< 0.005, **** P-value< 0.001

Furthermore, both basal and hyperinsulinemic FFA concentrations were significantly lower in caspase1-/- mice compared to control mice (basal: 0.69 ± 0.06 vs.0.89 ± 0.04, P-value=0.001; hyperinsulinemic :0.30 ± 0.02 vs. 0.41 ± 0.04; P-value=0.01), consistent with a reduction in lipolytic activity and an increase in insulin sensitivity of white adipose tissue in the absence of caspase-1. Other basal plasma lipids including triglycerides and cholesterol were not altered in caspase-1 deficient animals (data not shown). Since caspase-1 controls IL-1β activity, insulin tolerance was also analyzed in IL-1β-deficient animals. Insulin sensitivity was improved in IL-1β-deficient animals when compared to wild-type mice (Figure 4G). Finally, in line with the important role of NLRP3 in the activation of caspase-1, NLRP3-/- animals were more insulin sensitive compared to wild-type animals (Figure 4H). Noticeably, adipose tissue morphology in IL-1 and NLRP3-deficient animals was unchanged. Nevertheless, mtDNA content tended to be higher in NLRP3-/- animals although differences were not statistically significant (supplement Figure 1D).

Caspase-1 inhibition during obesity improves insulin sensitivity

The involvement of caspase-1 in the modulation of insulin resistance renders it an attractive therapeutic target in clinical conditions such as type 2 diabetes. In order to provide the proof-of-principle that caspase-1 inhibition can improve insulin sensitivity, ob/ob mice were treated with a chemical inhibitor of caspase-1 activity. Animals received orally 200mg/kg bodyweight caspase-1 inhibitor (Pralnacasan) or vehicle daily for two weeks. After two weeks of treatment, insulin tolerance tests were performed. Animals treated with the caspase-1 inhibitor showed a robust improvement of insulin sensitivity as compared to animals receiving vehicle only (Figure 5A). The effectiveness of caspase-1 inhibition was analyzed by measuring IL-1β protein levels in white adipose tissue. As expected, caspase-1 inhibition led to a significant reduction of IL-1β protein concentration in fat (Figure 5B). Noticeably, treatment of Ob/Ob animals with pralnacasan did not result in changes in faecal fat content or physical activity levels of the animals.

Interestingly, the improvement of insulin sensitivity was accompanied by an attenuated increase in bodyweight in animals receiving the caspase-1 inhibitor (Figure 5C) despite a similar daily food intake (Figure 5D). The percentage of epidydimal white adipose tissue in ob/ob animals receiving the inhibitor tended to be lower as compared to animals receiving vehicle only (P-value=0.06) suggestive for changes in lipid storage and lipogenesis in white adipose tissue (Figure 5E).

Because lipid composition may be involved in the effects of caspase-1 on adipose

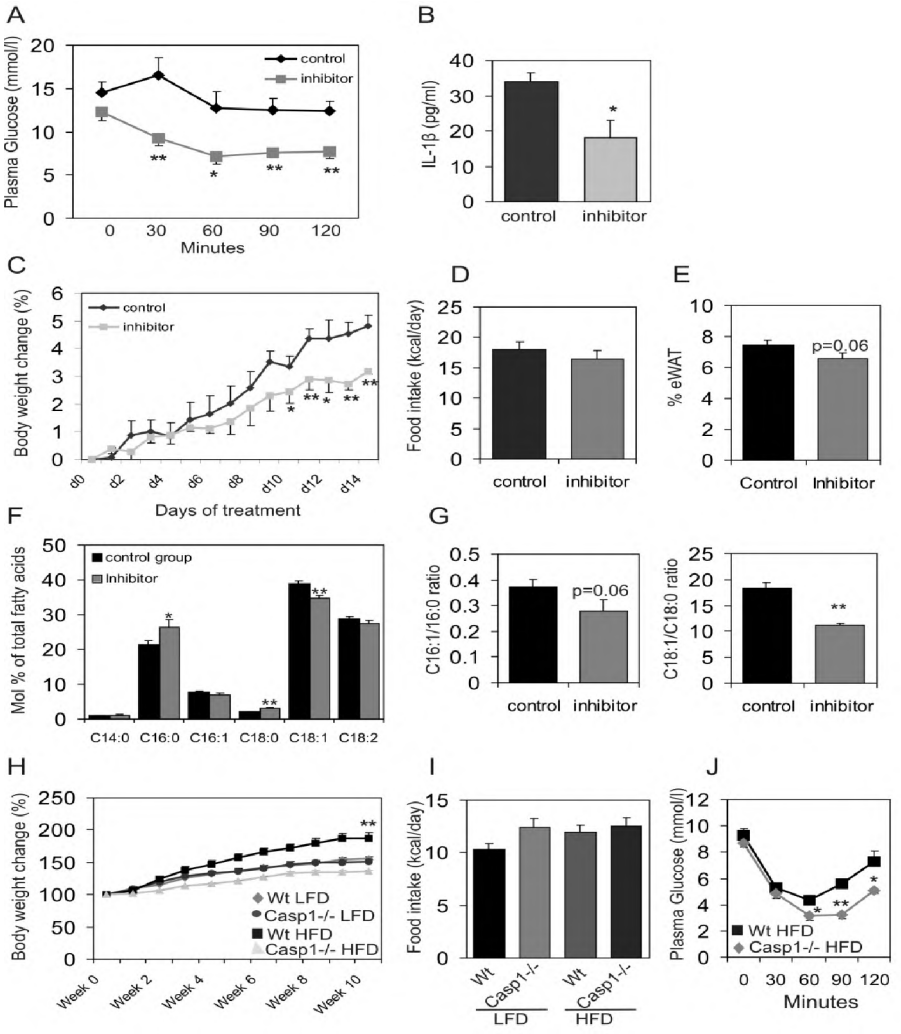

Figure 5 **Caspase-1 blockage improves insulin sensitivity**. A, Insulin tolerance test in male Ob/Ob animals (2 months of age at start of intervention) orally treated with a chemical caspase-1 inhibitor (Pralnacasan) or vehicle for two weeks (n=5 per group). B, IL-1β concentrations (pg/ml) measured in total white adipose tissue of Ob/Ob mice receiving vehicle or a caspase-1 inhibitor (n=5 per group). C, Body weight change (in %) during oral treatment of Ob/Ob animals with vehicle or a caspase-1 inhibitor (n=5 per group). D, Food intake (kcal/day) of Ob/Ob animals orally treated with a chemical caspase-1 inhibitor or vehicle for two weeks. E, percentage of epididymal white adipose tissue. F, lipid composition of white adipose tissue. G, Desaturation indexes in vehicle or caspase-1 inhibitor treated animals. H, Bodyweight development of wild-type or caspase-1-/- animals (2 months of age at start of intervention) fed a low fat diet or high fat diet for 10 weeks (n=10 per group). I, Food intake (kcal/day) of wild-type or caspase-1-/- fed a LFD or HFD. J, Insulin tolerance test in wild-type and caspase-1-/- animals fed a HFD for 10 weeks (n=5 per group). Data are presented as mean ± sem. Asterisks depict statistically significant differences between control and experimental groups.* P-value<0.05, ** P-value<0.005

tissue, the general lipid composition of the white adipose tissue was analyzed. As shown in figure 5F, the amounts of C16:0 (palmitic acid) and C18:0 (stearic acid) present in white adipose tissue were significantly higher in animals receiving the inhibitor whereas the concentration of C18:1 (oleic acid) was lower. Finally, the ratios of C16:1/C16:0 and C18:1/C18:0, indicative of desaturation through SCD-1 activity, were lower in Ob/Ob animals treated with the inhibitor (Figure 5G) suggestive for a decrease in lipogenesis.
Inasmuch no changes were observed in the ratios of C18:0/C16:0 and C16:1/C18:1 (data not shown), elongase activity appeared to be unchanged. Noticeably, fatty acid composition and ratios of C18:1/C18:0 and C16:1/C16:0 in plasma samples were unchanged. Plasma FFA levels were unchanged in ob/ob animals fed the inhibitor as compared to the control animals (0.18mM ± 0.04 vs. 0.18mM ± 0.009).
The effect of caspase-1 on the development of obesity and insulin resistance was analyzed using a diet intervention. 10 weeks of HFD feeding led to significantly less bodyweight gain in caspase-1-/- mice as compared to HFD fed wild-type animals (Figure 5H) despite a similar food intake (Figure 5I). Moreover, insulin sensitivity as measured by an ITT test was significantly higher in caspase-1-/- animals compared to wild-type mice fed the HFD (Figure 5J).

Absence of caspase-1 increases fat oxidation rate

Inasmuch the inhibition of caspase-1 attenuates bodyweight gain despite a similar food intake, caspase 1-/- and C57Bl/6 wild-type mice were subjected to indirect calorimetry analysis using metabolic cages to determine their energy expenditure. Food intake and respiratory gas exchange was measured at 7 minute intervals for a period of 60 hours. Respiratory gas exchange was calculated from oxygen consumption and carbon dioxide production, and analyzed separately for diurnal and nocturnal periods to distinguish between periods of low (diurnal) and high (nocturnal) physical activity. In line with our previous results, food intake was similar in both groups (wild-type, 4.99 ± 0.26g/day; caspase-1-/-, 4.62 ± 0.15g/day; *P*-value=0.3). Fecal output (wild-type, 1.4 ± 0.07g/day; caspase-1-/-, 1.4 ± 0.07g/day; *P*-value=0.9) as well as fecal fat content (steatocrit %: wild-type, 7.13 ± 0.38; caspase-1-/-, 8.02 ± 0.49; *P*-value=0.17) were not different between both genotypes.
Total energy expenditure did not differ between groups during the nocturnal period (wild-type, 0.54 ± 0.03kcal/h; caspase-1-/-, 0.62 ± 0.03kcal/h; *P*-value=0.06), nor the diurnal period (wild-type, 0.45 ± 0.02kcal/h; caspase-1-/-, 0.45 ± 0.02kcal/h; *P*-value=0.9). Interestingly, diurnal RER values were significantly lower in caspase 1-/- animals (wild-type, 0.919 ± 0.012; caspase-1-/-, 0.885 ± 0.006) (Figure 6A). The lower RER values were translated into a higher absolute fat oxidation rate during the diurnal part of the day (wild-type, 0.09 ± 0.01kcal/h; caspase-1-/-, 0.13 ± 0.01kcal/h; *P*-value=0.02) (Figure 6B). Nocturnal relative and absolute fat oxidation rates as well as total diurnal and nocturnal carbohydrate oxidation rates did not differ between

groups (Figure 6B and C). Together, these data indicate that caspase-1-/- animals have higher fat oxidation rates compared to controls, while food intake and fecal output was equal between groups.

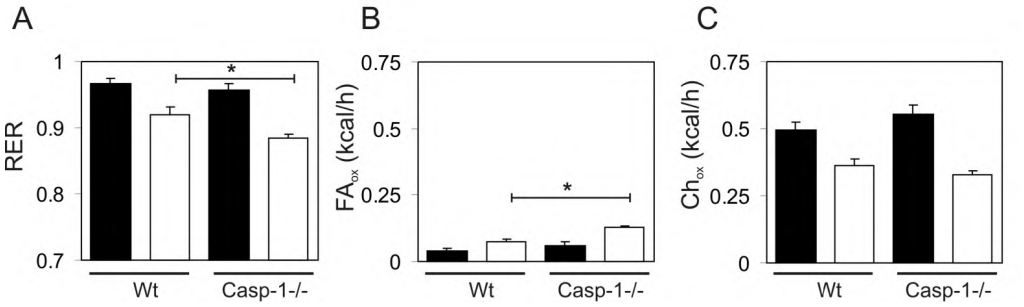

Figure 6 **Absence of caspase-1 augments fat oxidation rate**. A, Respiratory exchange rates, B, fat oxidation and C, carbohydrate oxidation levels in wildtype and caspase 1-/- animals. Black and white bars represent mean nocturnal and diurnal data ± SEM, respectively. * indicates *P*-value<0.05.

Discussion

In the present study we demonstrate that during differentiation and lipid accumulation, adipose tissue undergoes important pro-inflammatory changes represented by inflammasome and caspase-1 activation. These changes lead to a higher of IL-1β production and contribute to the induction of insulin resistance.

The importance of inflammation and infiltration of macrophages in the modulation of adipose tissue function and insulin resistance has been intensively studied over the last years (3;4). IL-1β has been previously linked to obesity-induced inflammation and the development of insulin resistance in adipose tissue (26;27). Our results demonstrate that caspase-1 activation in adipose tissue of obese animals takes place partly independent of macrophage infiltration, induces the release of IL-1β, and finally results in the development of insulin resistance through autocrine and paracrine effects. In the absence of caspase-1, adipocytes differentiate more efficiently and are more sensitive to insulin. We observed similar effects in the absence of the inflammasome component NLRP3 that has been previously demonstrated to mediate caspase-1 activation (9). In addition to robust changes in adipose tissue morphology, we also suggest that the absence of caspase-1 results in an increased mitochondrial energy dissipation reflected by an increase in PGC-1α and PGC-1β gene expression and enhancement of mtDNA content in white adipose tissue. Expression levels of PGC-1α and β in caspase-1 deficient animals are probably increased due to the absence of bio-active IL-1β (28). Assessment of additional metabolic parameters of caspase-1-/- animals revealed that, although total caloric expenditure as well as food intake and

fecal output did not differ between groups, absolute fat oxidation rates were higher in caspase-1-/- animals. Whereas fat oxidation comprised 20% of the total amount of diurnal caloric expenditure in wild-type animals, it contributed up to 30% in the caspase-1-/- animals. Ultimately, this results in a less positive fat balance and can explain the reduction in fat mass observed in caspase-1-/- animals.

In addition to activation of IL-1β, caspase-1 also cleaves pro-IL-18 to its bioactive form. In line with activation of caspase-1 in adipose tissue, IL-18 protein levels were increased in adipose tissue of HFD fed animals compared to mice fed the LFD. However, our in vitro data show that the effects of IL-18 on adipocyte differentiation and gene expression are minor as compared to IL-1β. In addition, it has been previously shown that aging IL-18-/- animals develop obesity and insulin resistance (8). Overall this suggests that, although IL-1β and IL-18 both serve as substrates of caspase-1, the effects of these cytokines on adipocyte function and insulin sensitivity appear to be opposite, and the absence of IL-1β masks the effects of IL-18.

Importantly, we show that caspase-1 deficient animals are more insulin sensitive, which strongly supports the concept of the involvement of the inflammasome/caspase-1 pathway in the development of insulin resistance associated with obesity. Additionally, we demonstrate that blockage of caspase-1 in obese and insulin resistant animals results in an improvement of insulin sensitivity and a smaller increase in total bodyweight. This reduction in weight gain is also reflected by a lower adipose tissue mass in animals receiving the caspase-1 inhibitor. The relatively small reduction in adipose tissue mass in animals receiving the inhibitor may be partly explained by the limited treatment period. Longer treatment of the animals with a caspase-1 inhibitor might lead to more pronounced differences in adipose tissue mass. In addition to the quantitative changes, we also observed qualitative changes in adipose tissue composition illustrated by a significantly lower desaturation in white adipose tissue of animals treated with the inhibitor. This suggests that SCD-1 activity that catalyzes the synthesis of monounsaturated fatty acids is lower after caspase-1 inhibition. Since SCD-1-/- animals have a reduction in adiposity due to enhanced lipid oxidation together with an improvement in insulin sensitivity (29), regulation of SCD-1 activity by caspase-1 in adipose tissue may partly explain our observations. Inasmuch SREBPs, that partly control SCD-1 activity, are processed and activated by caspase-1 (30), it may explain the reduction in desaturation index in adipose tissue of ob/ob animals after caspase-1 inhibition.

Hypothetically, the absence of caspase-1 in adipose tissue leads to an enhanced adipogenesis paralleled by an improvement in lipid oxidation due to enhanced mitochondrial function. The stimulation of adipogenesis in caspase-1-/- animals might primarily prevent lipid accumulation in non-fat tissues. In addition, it is unlikely that caspase-1

activation in adipose tissue will solely lead to IL-1β and IL-18 production since it has been reported that caspase-1 is able to cleave additional substrates (31). Adipocyte-specific proteins may also serve as caspase-1 substrates leading to functional changes in adipose tissue of obese animals. Interestingly, it has been shown that caspase-1 is able to cleave and inactivate PPARγ in adipocytes (32) that may also contribute to the detrimental effects of caspase-1 activation in adipose tissue of obese animals. Furthermore, adipose tissue-independent effects of caspase-1 may also contribute to the development of obesity-induced insulin resistance. Future studies are needed to resolve this issue and will shed more light on the mechanisms responsible for the resistance to diet-induced obesity in caspase-1-/- animals.

Beneficial effects of IL-1 blockade by the natural antagonist IL-1Ra have been recently reported in type 2 diabetes patients (7), and our data provides the rationale for proposing caspase-1 inhibition as a potential novel therapeutic target in conditions associated with obesity and/or insulin resistance.

One important question that remains to be answered regards the precise mechanisms leading to caspase-1 activation during accumulation of fat in the adipocytes. It is still conceivable that the influx of macrophages might influence the activation of adipocyte-specific caspase-1, although our in-vitro experiments showing caspase-1 activation in adipocytes in settings free of inflammatory cells suggest that adipocytes represent an important source of caspase-1. Nevertheless, our results do not rule out crosstalk between adipocyte-specific caspase-1 and production of IL-1β by macrophages in adipose tissue. Alternatively, lipids themselves or other mediators encountered in the obese patient (e.g. adipokines) could induce activation of caspase-1. Finally, glycemic conditions might induce caspase-1 activation in adipose tissue. Interestingly, hyperglycemic conditions have been shown to activate NLRP3 in pancreatic cells and a similar mechanism may exist in adipose tissue (33). Unraveling these molecular mechanisms is one of the important challenges for the near future.

In conclusion, our data reveal a novel metabolic function of the inflammasome/caspase-1 in adipose tissue. An increase in fat mass causes up regulation and activation of caspase-1 that counteracts the normal metabolic function of adipose tissue leading to insulin resistance. Inhibition of caspase-1 in obese and insulin resistant animals strongly improves insulin sensitivity together with changes in adipose tissue mass and composition. Importantly, the absence of caspase-1 results in an enhancement of overall fat oxidation rates. These findings suggest that pharmacological inhibition of caspase-1 in obese and/or patients with type 2 diabetes may restore the metabolic function of adipose tissue, and subsequently improve insulin sensitivity.

Acknowledgements

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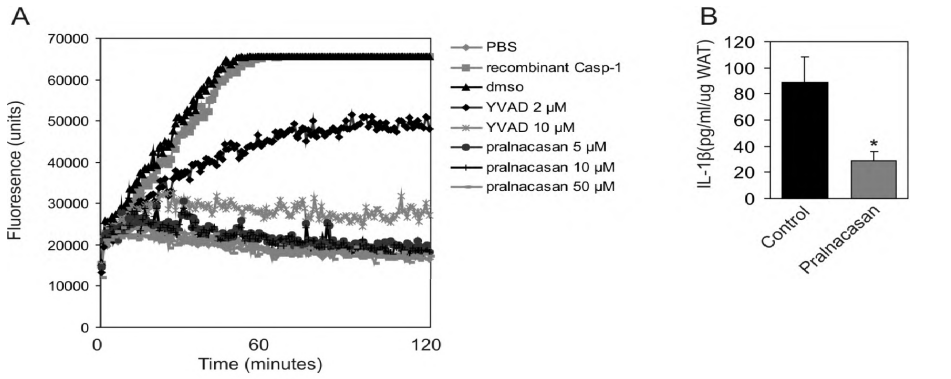
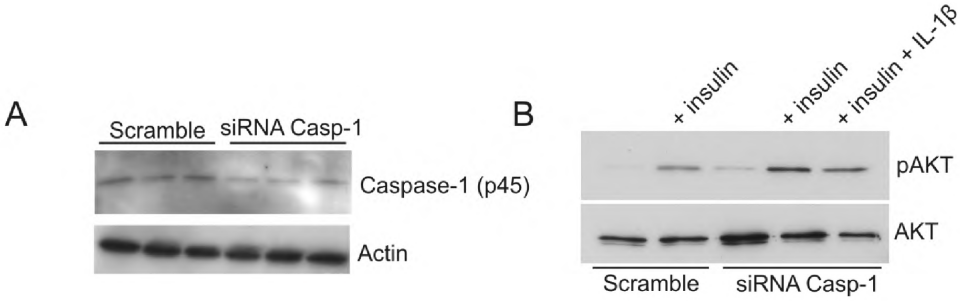
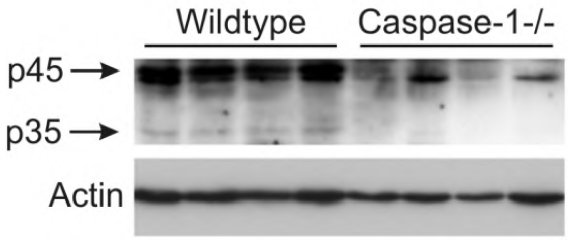
Supplemental data



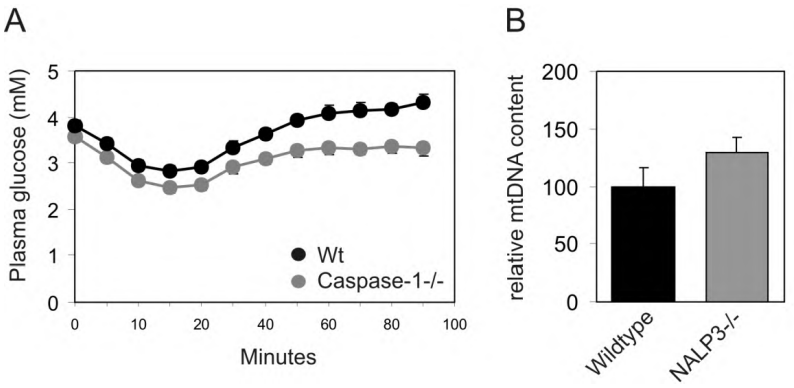
Supplemental figure 1 A, Assessment of the inhibition of caspase-1 by Yvad and Pralnacasan using a fluorometric activity assay. B, IL-1β production by human white adipose tissue after 24 hours of culture in the presence or absence of caspase-1 inhibitor Pralnacasan (100µM).



Supplemental figure 2 A, Protein levels total caspase-1 after 48 hours treatment of SGBS cells with scramble or caspase-1 targeted siRNA. B, pAKT levels in SGBS adipocytes after siRNA treatment targeted against caspase-1 and subsequent recombinant IL-1β exposure overnight.



Supplemental figure 3 Western blot analysis of caspase-1 protein levels in total white adipose tissue of wild-type and caspase-1-/- animals



Supplemental figure 4 A, Glucose values during euglycemic hyperinsulinemic clamp analyisi in wild-type (n=17) and caspase-1-/- animals (n=18), mean ± SEM. B, relative mtDNA content of white adipose tissue from wild-type and NALP3-/- animals.

Supplemental table 1

Animals	Glucose (mM)		Insulin (ng/mL)	
	Basal	Hyperinsulinemic	Basal	Hyperinsulinemic
Wild-type	4.12 ± 0.18	5.07 ± 0.24	0.29 ± 0.06	5.27 ± 0.63
Caspase-1-/-	4.00 ± 0.18	4.31 ± 0.19*	0.14 ± 0.04	5.16 ± 1.00

Plasma parameters under basal or hyperinsulinemic conditions in overnight fasted caspase-1 deficient and wild-type mice. Data are the means ± SEM. *, P-value<0.05 vs. wild-type.

Chapter 6

Hyperglycemia activates caspase-1 and TXNIP-mediated IL-1β transcription in human adipose tissue.

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Abstract

Introduction: Obesity is characterized by elevated levels of pro-inflammatory cytokines, including IL-1β, that contribute to the development of insulin resistance. In this study, we set out to investigate whether hyperglycemia drives IL-1β production and caspase-1 activation in mouse and human adipose tissue, thus inducing insulin resistance.

Research design and methods: Ob/Ob animals were used as a model to study obesity and hyperglycemia. Human adipose tissue fragments or adipocytes were cultured in medium containing normal or high glucose levels. Additionally, the role of thioredoxin interacting protein (TXNIP) in glucose-induced IL-1β production was assessed.

Results: TXNIP and caspase-1 protein levels were more abundantly expressed in adipose tissue of hyperglycemic Ob/Ob animals as compared to Wildtype mice. In human adipose tissue, high glucose resulted in a 10-fold up regulation of TXNIP gene expression levels (P-value<0.01) and a 10% elevation of caspase-1 activity (P-value <0.05), together with induction of IL-1β transcription (2-fold, P-value<0.01) and a significant increase in IL-1β secretion. TXNIP suppression in human adipocytes, either by an siRNA approach or a PPARγ agonist, counteracted the effects of high glucose on bioactive IL-1 production (P-value<0.01) mainly through a decrease in transcription levels paralleled by reduced intracellular pro-IL-1β levels.

Conclusions: High glucose activates caspase-1 in human and mouse adipose tissue. Glucose-induced activation of TXNIP mediates IL-1β mRNA expression levels and intracellular pro-IL-1β accumulation in adipose tissue. The concerted actions lead to enhanced secretion of IL-1β in adipose tissue that may contribute to the development of insulin resistance.

Introduction

Obesity is associated with a low-grade systemic inflammation, with elevated levels of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6, that contribute to the development of insulin resistance and progression to type 2 diabetes mellitus (T2DM). (1;2). The mechanisms that trigger the development of inflammation are currently unknown, although the adipose tissue has been viewed upon as the instigator of these effects (3;4). Earlier studies have shown that high glucose promotes the production of acute phase reactants (5) and pro-inflammatory cytokines by adipocytes (6). In addition, several studies have shown that hyperglycemia induces the production of IL-1β in different cell types including endothelial cells, monocytes, β-cells and other pancreatic islet cells (7-9). Although short time exposure to low concentrations of IL-1β enhances β-cell function, high glucose-induced islet IL-1β secretion is known to negatively interfere with insulin signalling and has cytotoxic effects on beta-cells leading to impaired insulin secretion (8;10). IL-1β is produced via cleavage of pro-IL-1β by caspase-1, a cysteine protease (11) that is activated by a protein complex named the inflammasome (12). Until recently, the mechanism by which high glucose induces IL-1β remained largely unknown. However, in a recent study Zhou et al. demonstrated a crucial role for thioredoxin interacting protein (TXNIP or vitamin D3 upregulated protein 1 (VDUP1)) during high glucose-mediated caspase-1 activation in mouse beta-cells, by direct interaction with the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR)-3 (NLRP3)-inflammasome (13). TXNIP is expressed in a wide variety of cell types including skeletal myocytes, pancreatic beta-cells, endothelial cells and adipocytes, and acts as an endogenous inhibitor of the reactive oxygen species (ROS) scavenging protein thioredoxin (14;15). TXNIP levels are elevated in subjects with T2DM (15) and its expression is induced by glucose-6-phosphate through an intracellular transcriptional complex of MondoA and Max-like protein X (16).
Here we investigate whether high glucose levels also drive TXNIP and caspase-1 activation in human adipocytes and intact adipose tissue and whether this may contribute to the production of IL-1β.

Research design and methods

Animal experiments
Blood glucose levels of five 8-week old male Ob/Ob C57/Bl6 and Wildtype C57/Bl6 animals (Jackson Laboratory) were determined after four hours of fasting. A glucose tolerance test and insulin tolerance test was performed in five 8-week old male caspase-1 -/- C57/Bl6 mice (average bodyweight: 23.6grams) and five age- and weight-matched wild-type C57/Bl6 animals. Epididymal white adipose tissue was used to

analyze protein levels of caspase-1 and TXNIP.

In-vitro and ex-vivo experiments with human adipose tissue
Intact adipose tissue fragments and cultured human preadipocytes from subcutaneous adipose tissue (n=6), obtained during plastic surgery, and the SGBS cell line were used to study the effects of high glucose levels. The protocol was approved by the hospital ethics committee and the tissue samples were collected after written informed consent. Adipose tissue fragments were directly cultured for 48 hours in DMEM supplemented with 10% fetal calf serum containing normal glucose levels (5mM) or high glucose levels (25mM). Pre-adipocytes were isolated from the adipose tissue according the procedure described by Rodbell et al. (17). Primary or SGBS preadipocytes were differentiated towards mature adipocytes using a standard adipogenic protocol (18). After 12 days of differentiation adipocytes were cultured under 5mM or 25mM glucose conditions. Mannose was added to cells cultured in the presence of 5mM glucose.

RNA isolation and PCR analysis
RNA was extracted from total adipose tissue or adipocytes using TRIzol reagent (Invitrogen, Carlstad, USA). cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio–Rad Laboratories, Hercules, CA). Real-time PCR was done using Power-SYBR® Green master mix and the 7300 Real-Time PCR system (Applied Biosystem, Warrington, UK). Expression of genes was normalized to β2M or 36B4 gene expression levels. Primer sequences are available upon request.

Protein analysis
Protein expression of TXNIP, caspase-1, β-actin and GAPDH were measured by western blotting. Antibodies were from Santa Cruz (caspase-1), Zymed (TXNIP), Abcam (NLRP3), Sigma-Aldrich (β-actin) and Calbiochem (GAPDH). Bioactive IL-1 secretion was quantified in a bioassay using the murine thymoma cell line EL4/NOB 1 that produces IL-2 in response to bioactive IL-1 (19). IL-2 and intracellular human pro-IL-1β levels were measured by Elisa (R&D). In short, medium collected from adipocytes or total adipose tissue cultured in the presence of 5mM or 25mM of glucose was added to NOB-1 cells. After 24 hours of incubation, medium was used for IL-2 measurements. The specificity of the NOB-1 assay to produce IL-2 in response to IL-1β was confirmed in this study (Supplemental figure 1).

Caspase-1 activity assay
Caspase-1 activity in adipocyte lysates was determined with a caspase-1 fluorometric kit (Biovision) following the cleavage of 50μM peptide YVAD-AFC. The fluorescence of the cleaved substrate was measured every 90 seconds using a fluorometer (Polarstar BMG, fluostar galaxy).

Small interference (si) RNA
To specifically suppress TXNIP expression in differentiated adipocytes, cells were transfected (X-tremeGENE siRNA Transfection Reagent, Roche) with siRNA against TXNIP (Thermo Scientific). As a non-specific control, scrambled siRNA (Thermo Scientific) was used.

Statistical analysis
Variables are expressed as means ± SD. One-way ANOVA , the Wilcoxon rank test and Student's paired t-test were used to analyze statistical significance. Two-tailed *P*-value<0.05 was considered significant. All statistic analysis were performed using SPSS software (version 16.0; SPSS Inc.,Chicago, IL).

Results

To determine whether hyperglycemic conditions induce caspase-1 and TXNIP in adipose tissue *in-vivo*, we used the Ob/Ob animal model which is characterized by, obesity, insulin resistance and hyperglycemia (Figure 1A). Both the inactive pro-caspase-1 (p45) and the active caspase-1 (p35) protein levels were up-regulated in Ob/Ob mice compared to the normoglycemic Wildtype animals (Figure 1B). In parallel with caspase-1 activation, TXNIP protein levels were elevated in adipose tissue of Ob/Ob animals. Interestingly, the absence of caspase-1 led to an improvement of glucose tolerance (Figure 1C) and insulin sensitivity (Figure 1D). Supported by the observation that TXNIP-/- animals are more insulin sensitive (20), these *in-vivo* results strongly suggest a link between TXNIP and caspase-1 activation during hyperglycemic conditions in adipose tissue that contributes to the development of insulin resistance.

Next, we studied the effects of high glucose on caspase-1 activation and the production of IL-1β in human adipose tissue. Treatment of adipose tissue with 25mM glucose increased the gene expression levels of pro-inflammatory mediators including IL-6 and IL-8 (*P*-value<0.01) together with a significant 4-fold reduction in PPARγ expression levels (Figure 2A). Moreover, IL-1β gene expression levels were also elevated upon short- or long term stimulation of adipose tissue and adipocytes with 25mM of glucose (7.5 fold; *P*-value<0.01, 4 fold; *P*-value<0.06, respectively), whereas IL-18 levels were unaffected (Figure 2B and 2C). In line with activation of IL-1β transcription levels, both the intracellular content of pro-IL-1β and secretion of bioactive IL-1 were significantly elevated after exposure of adipocytes or intact adipose tissue to 25mM of glucose (Figure 2D and 2E). Since IL-1 production depends on caspase-1 activation, we determined caspase-1 activity levels in adipocytes treated with 5mM or 25mM of glucose. As shown in figure 2F, a significant increase in caspase-1 activity was observed in primary human adipocytes treated with 25mM of

glucose for 48h, compared to adipocytes treated with 5mM of glucose. In line with the enhanced caspase-1 activity, high glucose levels led to an increase in NLRP3 protein levels in primary human adipocytes (Figure 2G). Although only a trend was observed (*P*-value=0.07), our results imply that NLRP3 is one of the signaling molecules that translate high glucose levels into caspase-1 activation. Noticeably, similar results were obtained using human adipose tissue explants (data not shown). Overall, these results suggest that high glucose induces IL-1β transcription, activation of caspase-1 and secretion of IL-1 in human adipose tissue.

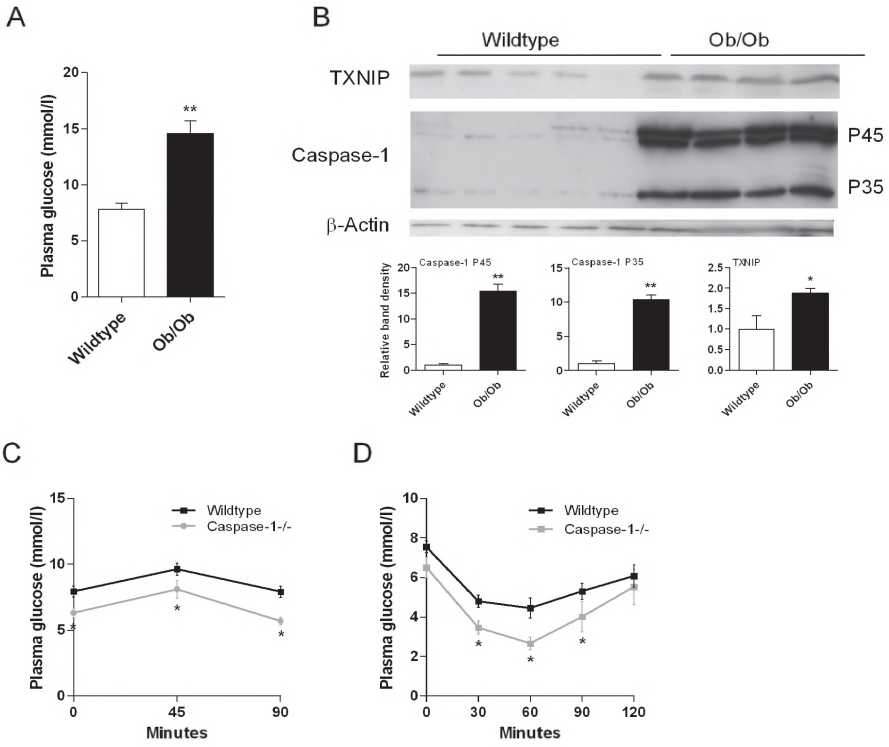

Figure 1. **TXNIP and caspase-1 protein levels are increased in the adipose tissue of Ob/Ob mice.** A. Plasma glucose levels in fasted wild-type mice and Ob/Ob mice (*n*=5 per group). B. Western blot images and quantification of TXNIP, pro-caspase-1 (p45) and active caspase-1 (p35) protein levels in the epididymal adipose tissue of wild-type mice and Ob/Ob mice (*n*=5 per group). C. A glucose tolerance test was done using fasted wild-type and caspase-1-/- animals (*n*=5 animals per group). D. An insulin tolerance test was performed in fasted wild-type and caspase-1-/- animals (*n*=5 animals per group). * = p-value < 0.05, ** = p-value < 0.01 using a Student's T-test.

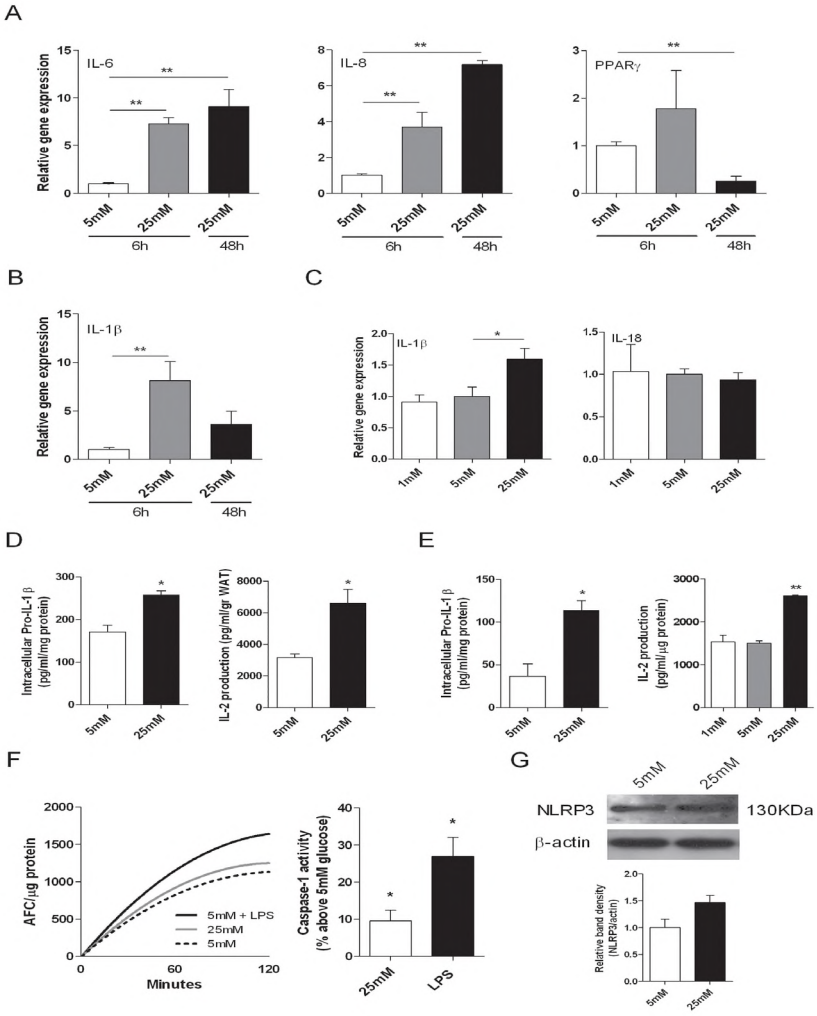

Figure 2. **Hyperglycemia induces pro-inflammatory gene expression and results in an increased production of IL-1 by intact adipose tissue and adipocytes.** A. IL-6, IL-8 and PPARγ gene expression levels in human intact adipose tissue (*n*=3) after 6h or 48h of glucose treatment. B. IL-1β gene expression levels in human adipose tissue (*n*=3) treated with glucose for 6h or 48h. C. IL-1β and IL-18 gene expression levels in human primary adipocytes (*n*=3) treated with various concentrations of glucose. D,E. Intracellular pro-IL-1β levels measured in lysates from intact human adipose tissue (D) or human primary adipocytes (E) treated with 5mM or 25mM glucose for 48 hours (*n*=4) and IL-2 production from NOB-1 cells after exposure to medium from intact human subcutaneous adipose tissue of three different donors treated with 5mM or 25mM of glucose for 48 hours. F. Caspase-1 activity assay in primary human adipocytes treated with 5mM, 25mM of glucose or LPS (10ng/ml) for 48 hours. The left graph displays the results of one representative experiment. The right graph displays the average results of *n*=6 experiments. G. NLRP3 protein expression levels in human primary adipocytes treated with 5mM or 25mM of glucose for 48 hours. A representative western blot and quantification of the results (*n*=3) are shown. * = p-value<0.05, ** = p-value<0.01 using an one-way ANOVA test (Figures A, B and C), Student's T-test (figures D and E) or a Wilcoxon rank test (Figure F; right graph).

To assess whether high glucose induces TXNIP in human adipose tissue and adipocytes to a similar extent as observed in Ob/Ob animals, we first studied the regulation of TXNIP during the differentiation of human preadipocytes towards fully mature adipocytes. Whereas TXNIP is expressed in human preadipocytes, expression levels rapidly increased during differentiation towards mature adipocytes both at gene and protein expression levels (Figure 3A).
In addition to caspase-1, TXNIP protein levels were detectable in total human subcutaneous adipose tissue and tended to increase in parallel with BMI (Figure 3B).
As adipose tissue is composed of both adipocytes and non-adipocyte cells, including inflammatory cells, we determined gene expression levels in both fractions. Purity of the different fractions was confirmed with the markers adiponectin (adipocyte-specific) and F4/80+ (macrophage-specific) (Supplemental Figure 2). Whereas TXNIP is expressed in the stromal vascular fraction (SVF), expression levels were 2.5 times higher in adipocytes (Figure 3C). As shown in figure 3D and E, glucose was a potent regulator of TXNIP mRNA expression in both isolated adipocytes and adipose tissue explants. Further, treatment of adipocytes with 5mM 2-deoxyglucose leading to a chronic activation of the MondoA:Mlx, resulted in a more pronounced activation of TXNIP gene expression (Figure 3D and 3E). In line with these transcriptional changes, protein levels of TXNIP varied in response to different glucose concentrations added to adipocytes or intact adipose tissue (Figure 3F and G).

Since high glucose potently activates TXNIP expression in adipocytes (Figure 3) and TXNIP has been shown to induce IL-1β secretion from mouse pancreatic islets cells in response to elevated glucose via activation of caspase-1 (13), we investigated whether TXNIP mediates high glucose-induced IL-1 production in human adipocytes using siRNA targeted against TXNIP. In adipocytes exposed to 25mM of glucose, successful knockdown of TXNIP by siRNA (Figure 4A) led to a significant reduction in bioactive IL-1 production towards basal IL-1 levels induced by 5mM glucose (Figure 4B). Additionally, IL-1β transcription and intracellular levels of pro-IL-1β were reduced upon siRNA-mediated depletion of TXNIP (Figure 4C). Whereas high glucose treatment increased caspase-1 activity levels in adipocytes (Figure 2F), siRNA mediated knockdown of TXNIP had no effect on caspase-1 activation (Figure 4D). Interestingly, reducing TXNIP gene expression with the PPARγ agonist Rosiglitazone in adipocytes and adipose tissue explants exposed to 25mM glucose for 48 hours (Figure 4E), also led to a significant decrease in intracellular pro-IL-1β and bioactive IL-1 production (Figure 4F and 4G). These results demonstrate that TXNIP contributes to high glucose-induced IL-1 release by modulating the transcription of IL-1β and the intracellular pool of pro-IL-1β.

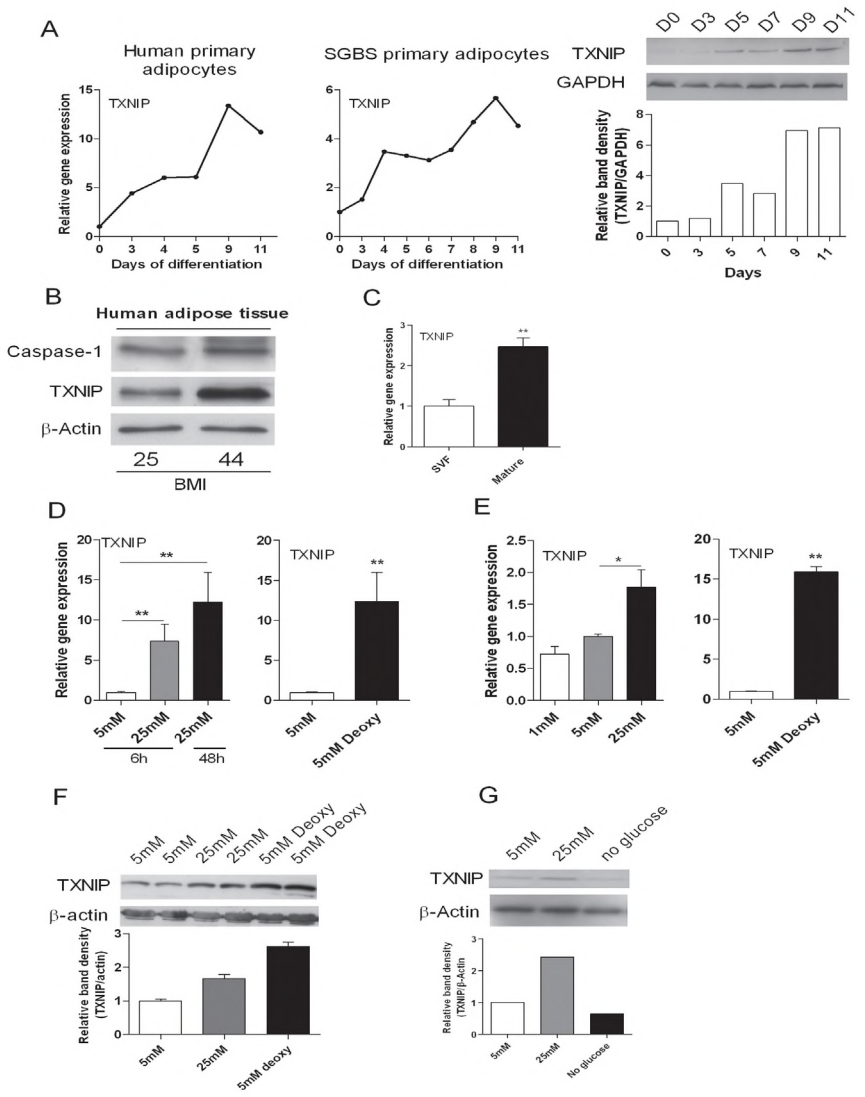

Figure 3. **TXNIP is present in human adipose tissue and adipocytes and is regulated by glucose.** A. TXNIP gene and protein expression levels during adipocyte differentiation of human primary adipocytes or human SGBS adipocytes. B. Western blot images of caspase-1 and TXNIP protein levels in total human subcutaneous adipose tissue (n=2). C. Relative gene expression levels of TXNIP in mature adipocytes or the stromal vascular fraction (SVF) isolated from human adipose tissue (n=3). D. Gene expression levels of TXNIP in intact adipose tissue treated with various concentrations of glucose (n=3). E. Gene expression levels of TXNIP in human primary adipocytes treated with various concentrations of glucose (n=3). F. Protein levels of TXNIP in human adipose tissue after 5mM glucose, 25mM glucose or 5mM deoxyglucose treatment for 48 hours (n=2). G. Protein levels of TXNIP after glucose starvation (no glucose), 5mM glucose and 25mM glucose for 48 hours in human primary adipocytes. * = p-value<0.05, ** = p-value<0.01 using an one-way ANOVA test (Figures D and E) or a Student's T-test (Figure C).

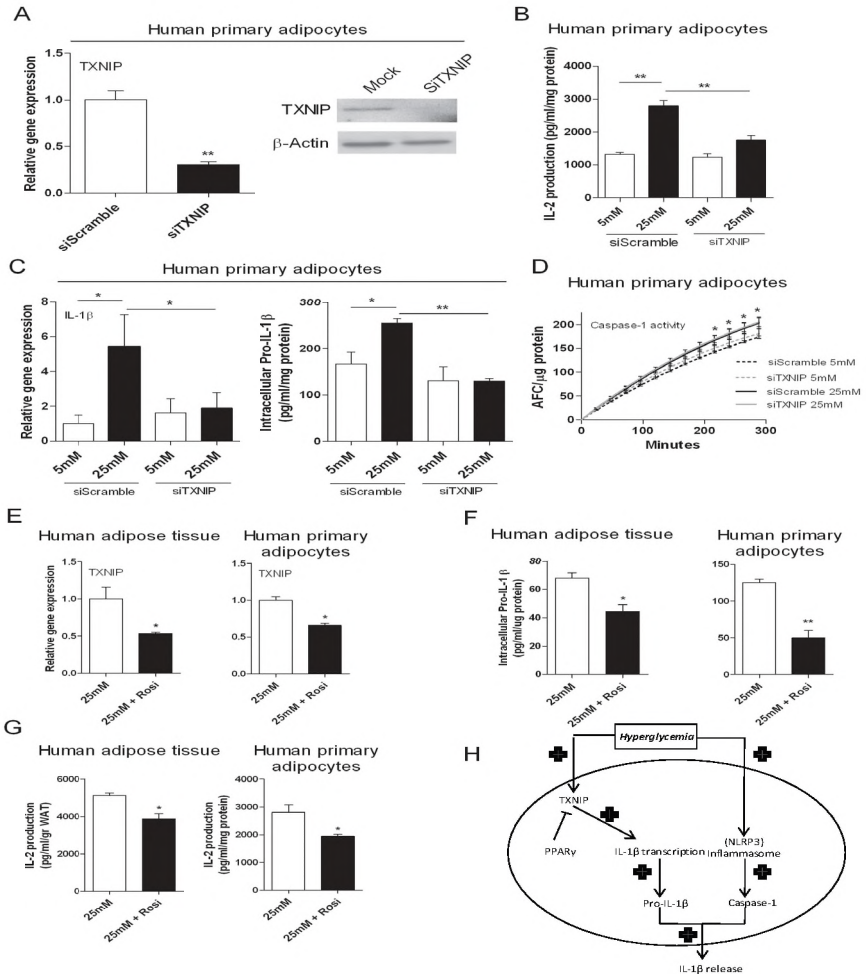

Figure 4. **TXNIP reduction results in a decline of high glucose-induced IL-1 production by modulating IL-1β gene expression.** A. Gene and protein expression levels of TXNIP after siRNA treatment against TXNIP in human primary adipocytes (*n*=6). B. IL-2 production from NOB-1 cells after exposure to medium from human primary adipocytes transfected with TXNIP siRNA and treated with 5mM or 25mM glucose for 48 hours (*n*=6). C. IL-1β mRNA expression and intracellular pro-IL-1β levels in TXNIP siRNA treated adipocytes exposed to 5mM or 25mM glucose for 48 hours (*n*=4). D. Caspase-1 activity assay in TXNIP siRNA treated human primary adipocytes exposed to 5mM or 25mM glucose for 48 hours (*n*=3). E. TXNIP gene expression levels in intact human adipose tissue and primary adipocytes exposed to 25mM glucose for 48 hours with or without Rosiglitazone (10µM) treatment for 24 hours (*n*=3). F. Intracellular pro-IL-1β levels measured in lysates of intact human adipose tissue and primary adipocytes exposed to 25mM glucose for 48 hours with or without Rosiglitazone (10µM) treatment for 10 hours (*n*=3). G. IL-2 production from NOB-1 cells after exposure to medium from intact adipose tissue and human primary adipocytes treated with 25mM glucose for 48 hours with or without Rosiglitazone (10µM) stimulation for 10 hours (*n*=4). H. Role of TXNIP in hyperglycemia-induced release of IL-1β from adipose tissue. * = *P*-value < 0.05, *** = *P*-value < 0.01 using an one-way ANOVA test (Figures B and C) or a Student's T-test (Figure D, E, F, G).

Discussion

Several studies show that IL-1β deteriorates peripheral insulin sensitivity and inhibits insulin production by the pancreas (8;10). The notion that IL-1β is relevant in human (patho)physiology is supported by the finding that blocking of IL-1 signaling pathways in T2DM patients by treatment with IL-1 receptor antagonist Anakinra improves glycemic control (21). However, the precise mechanisms leading to an enhanced production of IL-1β in the "inflamed" adipose tissue over the course of development of insulin resistance have remained obscure. The importance of hyperglycemia-induced insulin resistance in T2DM patients is well accepted, although no current explanation is available for this process. The stimulatory role of hyperglycemia on IL-1β production is however known for several years (7-9). The combination of this knowledge with the recent discovery of the important effect of IL-1β on the induction of insulin resistance (22) directed us to the hypothesis that hyperglycemia induces insulin resistance in adipose tissue through activation of caspase-1 and IL-1β secretion. Moreover, Zhou et al have recently uncovered TXNIP as an essential mediator of hyperglycemia-induced caspase-1-dependent IL-1β production in the mouse pancreatic β-cell (13), and we explored whether TXNIP could mediate a similar effect in human and mouse adipocytes. Our data clearly show that adipose tissue-resident caspase-1 and TXNIP are activated in both hyperglycemic Ob/Ob animals and in human adipose tissue treated with 25mM of glucose, resulting in an increased IL-1β production. Both the absence of caspase-1 and TXNIP (20) significantly improved insulin sensitivity and resulted in lower plasma glucose levels. Together, these findings strengthen the concept that aberrant IL-1β production via caspase-1 underlies the pathophysiology of T2DM in different cellular sources, linking this disease to auto-inflammatory syndromes.

The glucose-responsive TXNIP gene has been previously linked to diabetes and insulin resistance, since diabetic patients expressed consistently higher TXNIP mRNA gene levels in skeletal muscles (15), while animals lacking TXNIP displayed a hypoglycemic, hypoinsulinemic phenotype (23). Furthermore, it has been shown that PPARγ negatively regulates TXNIP expression in adipose tissue (24). In agreement, we showed that activation of PPARγ by its agonist Rosiglitazone led to reduced TXNIP levels together with a decline in IL-1β. The elucidation of the exact molecular mechanisms through which TXNIP contributes to enhanced IL-1β production by the adipose tissue will need further study. Whereas TXNIP has been shown to directly controls caspase-1 activation in pancreatic beta cells (13), our results suggest that, at least in adipocytes, TXNIP mainly regulates IL-1β mRNA transcription levels and intracellular pro-IL-1β pools and does not directly affect caspase-1 activity levels. This discrepancy may largely be explained by cell-specific differences in adipocytes vs. pancreatic beta cells. In line with our results, Masters et al. reported no changes in

caspase-1 activation upon TXNIP depletion in macrophages. However, these authors also failed to detect differences in IL-1β production upon stimulation with ATP and uric crystals in Wildtype vs. TXNIP-/- macrophages (25). This could be explained by the constitutive large expression of TXNIP in macrophages that is not influenced by high levels of glucose. In contrast, TXNIP does play a role in adipocyte glucose homeostasis and elevated levels of glucose potently induce TXNIP expression in adipocytes (15).

Since excessive production of reactive oxygen species (ROS) has been found to increase IL-β activity and may contribute to the toxic effect of high glucose on β-cells (26), future studies will need to reveal a possible role of TXNIP- induced oxidative stress induced by high glucose in mediating the release of IL-1β by adipose tissue. In addition, it would be interesting to investigate whether other metabolic stress signals that are elevated during the development of insulin resistance bear the potential to induce caspase-1.

In conclusion, adipocyte-specific TXNIP up regulation induced by hyperglycemia may contribute to a sustained pro-inflammatory state and hyperglycemia-associated insulin resistance partly through inducing IL-1β transcription. Furthermore, elevated levels of glucose increased the activation of caspase-1 in adipocytes. Although more efforts are needed, the high glucose-induced increase in NLRP3 protein levels in primary human adipocytes suggests involvement of the NLRP3-inflammasome in mediating caspase-1 activation during hyperglycemic conditions. Therefore, we suggest that, at least in adipose tissue, both activation of TXNIP and caspase-1 during hyperglycemic conditions lead to an enhanced production of IL-1β (Figure 4H). In this way, TXNIP links hyperglycemia to increased IL-1β production that may result in insulin resistance at the level of the adipose tissue. As a consequence, inhibition of TXNIP may represent a novel therapeutic target in the treatment of insulin resistance that will ameliorate adipose tissue functioning.

Acknowledgements

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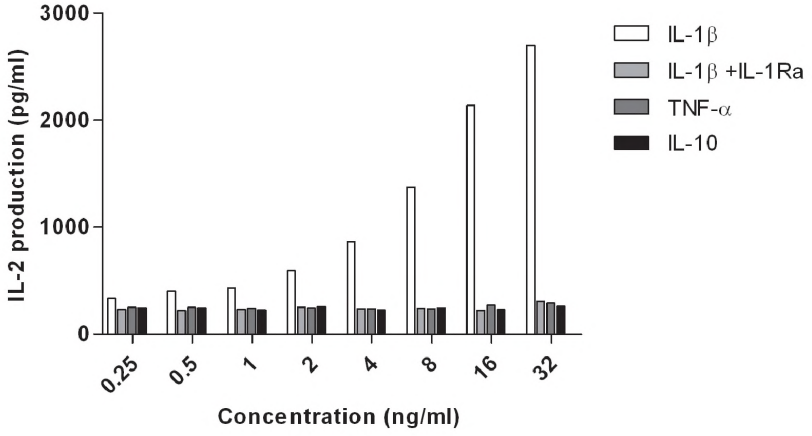
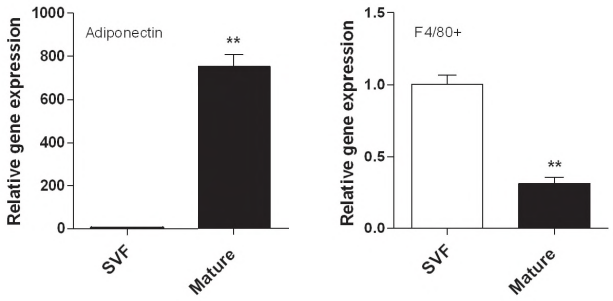
Supplemental data



Supplemental Figure 1. **Specificity of the NOB-1 bioassay**. The specificity of the NOB-1 bioassay was tested by incubation of NOB-1 cells with recombinant IL-1β (10ng/ml), TNFα (10ng/ml), IL-10 (10ng/ml) and IL-1Ra (15ug/ml).



Supplemental Figure 2. **Purity of adipose tissue fractioning.** Analysis of adiponectin and F4/80+ gene expression levels in the SVF and mature adipocyte fraction of subcutaneous adipose tissue. ** = p-value<0.01 using a Student's T-test.

Chapter 7

The inflammasome and caspase-1 activation: a new mechanism underlying increased inflammatory activity in human visceral adipose tissue

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Endocrinology (Conditionally accepted)

Abstract

The immune competent abdominal adipose tissue, either stored viscerally (VAT) or subcutaneously (SAT), has been identified as a source of IL-1β and IL-18. To become active, the pro-forms of these cytokines require processing by caspase-1, which itself is mediated by the inflammasome. In this descriptive study, we investigate the expression of inflammasome components and caspase-1 in human fat and determine whether caspase-1 activity contributes to the enhanced inflammatory status of VAT.

Paired SAT and VAT biopsies from ten overweight subjects (BMI: 25-28 kg/m^2) were used to study the cellular composition and the intrinsic inflammatory capacity of both adipose tissue depots.

The percentage of CD8$^+$ T-cells within the lymphocyte fraction was significantly higher in VAT compared to SAT (41.6% vs. 30.4%; *P*-value<0.05). Adipose tissue cultures showed a higher release of IL-1β (10-fold; *P*-value<0.05), IL-18 (3-fold; *P*-value<0.05), IL-6 and IL-8 (3-fold; *P*-value<0.05 and 4-fold; *P*-value<0.05, respectively) from VAT compared to SAT that was significantly reduced by inhibiting caspase-1 activity. In addition, caspase-1 activity was 3-fold (*P*-value<0.05) higher in VAT compared to SAT, together with an increase in the protein levels of the inflammasome members ASC (2-fold; *P*-value<0.05) and NLRP3 (2-fold; ns). Finally, caspase-1 activity levels were positively correlated with the percentage of CD8$^+$ T-cells present in adipose tissue. Our results show that caspase-1 and NLRP3 inflammasome members are abundantly present in human VAT. The increased intrinsic caspase-1 activity in VAT represents a novel and specific inflammatory pathway that may determine the pro-inflammatory character of this specific depot.

Introduction

Chronic low-grade inflammation has now been recognized as one of the key steps in the pathogenesis of obesity-induced insulin resistance and type 2 diabetes mellitus. The metabolically active abdominal adipose tissue secretes a wide variety of cytokines, that may promote the development of peripheral insulin resistance (1). Obesity-induced enlargement of adipose tissue is accompanied by elevated plasma levels of cytokines, including IL-6, IL-8, IL-1β, IL-18 and TNFα that affect insulin sensitivity in peripheral tissues (2-6). It has been suggested that especially visceral adipose tissue (VAT), rather than subcutaneous adipose tissue (SAT), contributes to the elevated circulating levels of inflammatory cytokines in obese individuals (7;8) that may be attributed to enhanced influx of immune cells including macrophages, monocytes, and B- and T-cells (9;10). However, efforts to identify possible mechanisms underlying the enhanced inflammatory capacity of VAT compared to SAT are scarce.

Recently, it was shown that the Toll-like receptor (TLR)-4 inflammatory pathway is activated in the adipose tissue during obesity and affects insulin responsiveness (11). The expression levels of multiple TLR family members were enhanced in VAT compared to SAT, suggesting that the TLR signaling pathway may contribute to an enhanced inflammatory capacity of VAT (12). IL-1β and IL-18 have been linked to the development of obesity and insulin resistance, and they partly originate from adipose tissue (4;13). In order to become active, the pro-form of IL-1β and IL-18 are processed by a cysteine protease named caspase-1. Activation of caspase-1 itself is mediated by a multiprotein complex entitled the inflammasome (14;15). Upon stimulation by exogenous (bacterial products) or endogenous (uric acids crystals, hyperglycemia, or cholesterol crystals) signals, formation of the inflammasome complex consisting of a NOD-like receptor (NLR) family member and the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) occurs (16-20). To date, activation and function of the NLRP3-inflammasome composed of NLRP3, the adaptor molecule ASC and caspase-1, is most fully characterized and responsible for recognition of invading pathogens and non-microbial molecules that eventuates into IL-1β and IL-18 production (21;22).

Inasmuch adipose tissue has been identified as a significant source of IL-18 and IL-1β, this suggests the presence of the NLRP-3 inflammasome machinery at the tissue level. Indeed, we have recently described that caspase-1 is well expressed in adipose tissue of obese animals and in human SAT (23;24), yet nothing is known about the NLRP3 inflammasome expression in human VAT compared to SAT. Therefore, we set out to study the presence of the NLRP3-inflammasome components and caspase-1 in human fat and to determine whether caspase-1 activity contributes to the enhanced inflammatory status of VAT versus SAT.

Methods

Subjects

Paired SAT and VAT (omentum) samples were obtained according a standardized procedure from ten patients (five females and five males) undergoing a cholecystectomy or an inguinal hernia surgery. Inclusion criteria were age between 40-60 years and body mass index (BMI) 25-28 kg/m^2. Subjects were normoglycemic and had a mean waist to hip ratio (WHR) of 0.90 (Supplementary table 1). Metabolic diseases, endocrine diseases and chronic and/or acute inflammatory diseases (high sensitivity C-reactive protein (hsCRP) above 1mg/L) were excluded. The tissue samples were collected after written informed consent and the protocol was approved by the ethical committee of the Radboud University Nijmegen Medical Centre.

Ex-vivo stimulation experiments with human adipose tissue

Intact human adipose tissue fragments from paired SAT and VAT were used to study the presence of the NLRP3 inflammasome components and the intrinsic caspase-1 activity as well as the cytokine release of IL-1β and IL-18 during a 24 hours culture using standard conditions (DMEM supplemented with 10% fetal calf serum containing 5mM glucose) with or without the addition of the caspase-1 inhibitor pralnacasan (100μM) (25).

Part of the freshly collected SAT and VAT samples were disaggregated using collagenase digestion to isolate mature adipocytes and the stromal vascular fraction (SVF). Purity of the two different fractions was confirmed with the markers adiponectin, leptin (adipocyte-specific) and CD45 (hematopoietic cell line marker (Supplementary figure 1). The separate cellular fractions were subsequently used for cell culture using standard conditions for 24 hours, FACS analysis and RNA isolation, followed by real-time PCR analysis.

RNA isolation and PCR analysis

RNA was extracted from total SAT and VAT or different adipose tissue cell fractions using TRIzol reagent (Invitrogen, Carlstad, USA). RNA concentration was determined using a NanoDrop™ (NanoDrop Technologies, Wilmington, USA) and cDNA synthesis was performed using the iScript ™ cDNA Synthesis Kit (Bio–Rad Laboratories, Hercules, CA). Real-time PCR was done using Power-SYBR® Green master mix and the 7300 Real-Time PCR system (Applied Biosystem, Warrington, UK). Expression of genes was normalized to β2M gene expression levels. Primer sequences are available upon request.

Protein analysis

Protein lysates from total adipose tissue of both depots were prepared to determine the presence of caspase-1 (Santa Cruz), NLRP3 (Abcam) and ASC (Abcam) by western

blotting. Secretion of IL-1β, IL-6, IL-8, IL-18, TNFα and adiponectin was analyzed by ELISA (R&D). Bioactive IL-1 secretion was quantified in a bioassay using the murine thymoma cell line EL4/NOB 1 that produces IL-2 in response to bioactive IL-1 (26). IL-2 levels were measured by ELISA (R&D).

Caspase-1 activity assay

Caspase-1 activity in total SAT and VAT protein lysates was determined with a caspase-1 fluorometric kit (Biovision) by measuring the cleavage of 50µl of the caspase-1 substrate YVAD-AFC. The fluorescence of the cleaved substrate was measured every 90 seconds using a fluorometer (Polarstar BMG, fluostar galaxy).

FACS analysis

SVFs of both SAT and VAT were analyzed by flow cytometry (FC500, Beckman Coulter). To this purpose, 100000 cells/100µl PBS + 1% BSA were incubated in three separate cocktails with the following conjugated monoclonal antibodies: anti-CD14 (Ph imm27074)-ECD, anti-CD45 (A 07785)-PCy5, anti-CD3 (A 07747)-PE, anti-CD8 (737659)-ECD, anti-CD4 (6604727)-ECD (Beckman Coulter), anti-F4/80 (ab60343-50)-FITC (Abcam). Blood contamination of the samples was prevented by treating the SVFs with an erythrocyte lysis buffer.

Statistical analysis

Data are presented as mean ± SEM. Comparisons between SAT and VAT parameters were calculated using the non-parametric Wilcoxon rank test. Correlations were determined using a Spearman correlation test. The cut-off for statistical significance was set at a *P*-value of 0.05 or below. All statistics were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL).

Results

Cellular composition of SAT and VAT

Since adipose tissue-resident macrophages represent potent inflammatory cytokine producers during obesity (27;28), we set out to study the macrophage content in the SVF of both SAT and VAT obtained by flow cytometry (Table 1). Our study revealed that the numbers of macrophages were equally distributed throughout SAT and VAT. Subsequent FACS analysis of the SVF of the adipose tissue to determine the cellular immune cell composition including monocytes and granulocytes, did not show significant differences between both fat depots. However, the percentage of $CD8^+$ (cytotoxic) T- lymphocytes was significantly increased in VAT compared to SAT, whereas the $CD4^+$ cell number was not different in both depots (Table 1). Supplementary figures 2A and B show representative dot plots of flow cytometry data of the different immune cells within the SVF of SAT and VAT, respectively.

Table 1

Immune cell composition of subcutaneous and visceral adipose tissue from seven subjects

Immune cells in SVF	SAT (%)	VAT (%)
Granulocytes (% of CD45+ cells)	29.0 ± 5.6	31.0 ± 6.5
Monocytes (% of CD45+ cells)	3.7 ± 0.8	2.8 ± 0.7
Macrophages (% of CD45+ cells)	5.7 ± 1.5	3.6 ± 1.3
Lymphocytes (% of CD45+ cells)	39.4 ± 6.0	47.3 ± 6.0§
T-cells (% of CD45+ cells)	29.2 ± 4.6	38.6 ± 5.4§
CD4 T-cells (% of CD45+ cells)	14.7 ± 3.0	16.4 ± 3.2
CD8 T-cells (% of CD45+ cells)	10.8 ± 2.0	16.6 ± 2.4†

Number of immune cells (percent) part of the innate immune system (granulocytes, monocytes, and macrophages) or adaptive immune system (total lymphocytes and T ($CD4^+$ or $CD8^+$) lymphocytes) in the SVF of SAT and VAT. Data are presented as mean ± SEM (n=7). §, P-value<0.05; †, P –value<0.01

Enhanced release of bioactive IL-1β and IL-18 from VAT compared to SAT

To examine the production capacity of IL-1β, IL-18 and other cytokines by VAT and SAT, total adipose tissue was brought into culture and cytokine production was measured after 24 hours. Interestingly, secretion of both total IL-1β and bioactive IL-1, as determined by ELISA and the NOB-1 bioassay, respectively, was significantly higher (P-value<0.05) in VAT compared to SAT. In addition, IL-18 production from VAT was also significantly enhanced (Figure 1A). The production of other pro-inflammatory cytokines including IL-6, IL-8 and IL-1Ra was also elevated in VAT compared to SAT explants (3-fold; P-value<0.05, 4-fold; P-value<0.05, and 2-fold; P-value<0.05, respectively) and secretion of adiponectin, a protein known for its insulin-sensitizing action (29), was reduced by VAT compared to SAT (P-value<0.05) (Figure 1B). Noticeably, secretion levels of the pro-inflammatory cytokine TNFα were comparable between VAT and SAT (Figure 1B).

To determine gene expression levels of different cytokines in VAT and SAT, qPCR analysis was performed. Whereas IL-1β gene expression levels were similar in both depots, IL-18 mRNA levels were significantly upregulated in VAT (Figure 2A). Gene expression levels of IL-6, IL-8 and adiponectin did not differ between both fat depots (data not shown). Fractioning of VAT into mature adipocytes and the SVF component revealed that IL-1β and IL-18 mRNA were significantly more expressed in the SVF (Figure 2B). In accordance with the gene expression profile, IL-1β production was elevated in the SVF compared to the mature adipocyte fraction in both fat depots. However, the production of IL-1β by mature adipocytes and SVF was higher in VAT compared to the correspondence fractions isolated from SAT (Figure 2C).

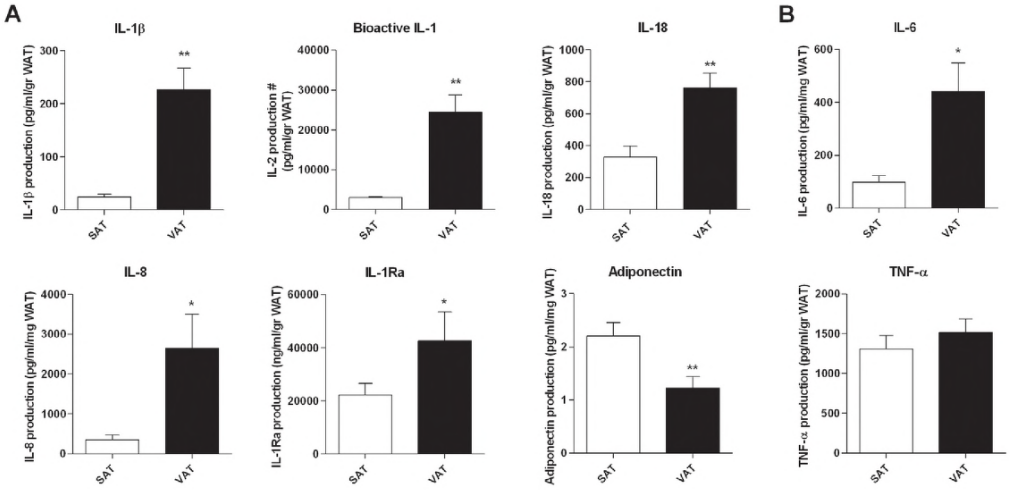

Figure 1. **Enhanced production of IL-1β, IL-18, IL-6, IL-8 yet not TNFα from VAT.** A. Secretion of IL-1β, bioactive IL-1 ([#] measured as IL-2 production from NOB-1 cells in response to bioactive IL-1), and IL-18 in intact SAT and VAT fragments cultured for 24hrs (n=5; BMI range 25-28). B. Secretion levels of IL-6, IL-8, IL-1Ra, adiponectin, and TNFα by VAT and SAT after 24hrs of culturing (n=5). [*] = P-value<0.05, [**] P-value<0.01 using a Wilcoxon rank test.

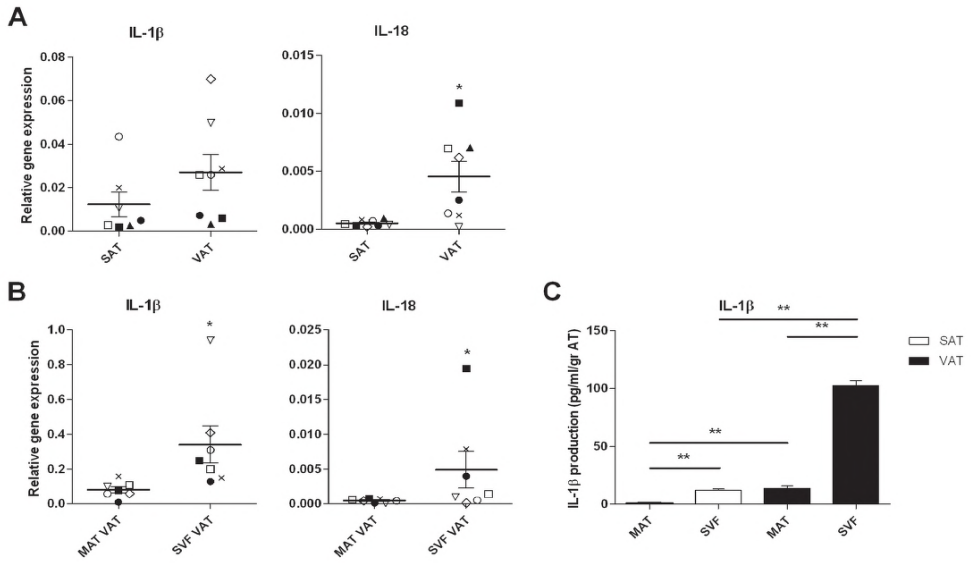

Figure 2. **Expression profile of IL-1β and IL-18 in VAT and SAT.** A. Relative IL-1β and IL-18 gene expression levels in total SAT and VAT (n=8). B. Relative IL-1β and IL-18 gene expression in mature adipocytes (MAT) and the SVF isolated from VAT (n=7; BMI range 25-28). C. IL-1β production by MAT and SVF isolated from one gram of SAT and VAT cultured for 24 hours (n=4). [*] = P-value<0.05, [**] P-value<0.01 using a Wilcoxon rank test.

Inflammasome expression and caspase-1 activation are increased in VAT compared to SAT

Inasmuch the adipose tissue is able to secrete IL-1β and IL-18 (Figure 1A), it suggests the presence of active caspase-1 in human adipose tissue. Indeed, the active form of caspase-1 was detectable in both fat depots (Figure 3A and 3B). Although caspase-1 gene expression was similar in both fat depots (data not shown), a 3-fold increase in caspase-1 protein levels was observed in the VAT samples from the ten study subjects as determined by Western-blot analysis (Figure 3A (western blot image from one subjects) and figure 3B (all subjects)). In addition, caspase-1 activity was enhanced in VAT compared to SAT as determined by a functional caspase-1 activity assay in freshly isolated adipose tissue from two patients (Figure 3C).

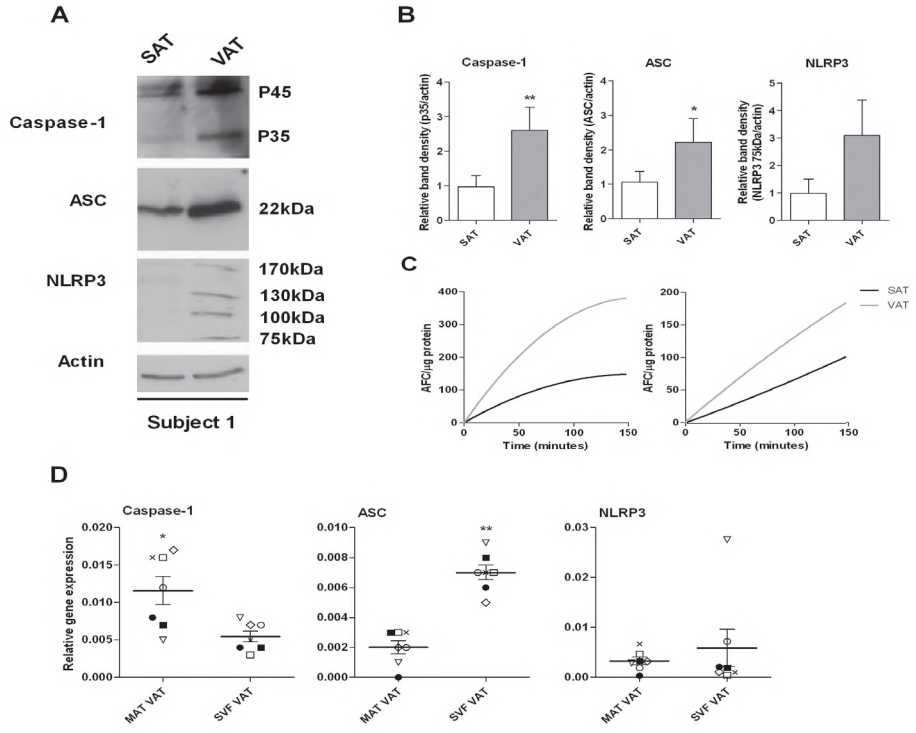

Figure 3. **Protein levels of ASC, NLRP3 and caspase-1 in VAT and SAT.** A. Caspase-1, ASC and NLRP3 protein levels in paired total SAT and VAT samples. Western blot images are shown for one subject. Several bands observed for NLRP3 represents the difference in length of the leucine-rich repeats, which forms a part of NLRP3 (53). B. Mean caspase-1, ASC and NLRP3 protein expression in paired SAT and VAT samples from ten subjects quantified by densitometry relative to actin protein levels. C. Functional caspase-1 activity assay in paired SAT and VAT of two different subjects. D. Relative caspase-1, ASC and NLRP3 gene expression in MAT and the SVF isolated from VAT (n=7) * = P-value<0.05, ** P-value<0.01 using a Wilcoxon rank test.

The abundant activity of caspase-1 in VAT (Figure 3A, B and C), implies the presence of the inflammasome machinery in human adipose tissue. Therefore, we tested whether the inflammasome components NLRP3 and ASC were present in the ten human VAT and SAT samples. Similar to caspase-1, ASC protein was detected in both depots, yet significantly upregulated in VAT (Figure 3A (western blot image from one subjects) and figure 3B (all subjects)). Due to a large inter-individual variation in NLRP3 protein levels between SAT and VAT, the expression levels of this protein only tended to be higher in VAT without reaching statistical significance. (Figure 3A and B). To investigate the cellular origin of the inflammasome components in human adipose tissue, qPCR analysis of fractionated VAT revealed that caspase-1 gene expression mainly originated from mature adipocytes, while ASC mRNA expression levels were higher in the SVF (Figure 3D). NLRP3 transcription levels were equally distributed between the two fractions of VAT (Figure 3D).

Blocking of caspase-1 inhibits cytokine release of VAT

To determine the potential of caspase-1 blockage to reduce the inflammatory trait of VAT, caspase-1 activity was blocked by the specific inhibitor pralnacasan. In figure 4A, the enhanced release of both IL-1β and IL-18 in VAT was significantly reduced when caspase-1 was blocked.

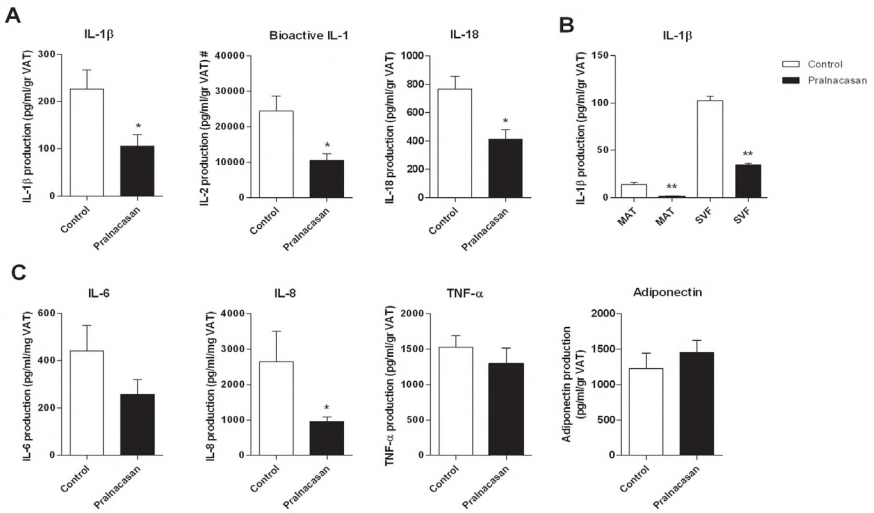

Figure 4. **Caspase-1 inhibition reduces production of IL-1β, IL-18, IL-6, IL-8 yet not TNFα from VAT.** A. Secretion of IL-1β, bioactive IL-1 ($^{\#}$ measured as IL-2 production from NOB-1 cells in response to bioactive IL-1), and IL-18 by intact VAT (n=5) cultured for 24 hours in the presence or absence of pralnacasan (100μM). B. IL-1β production by MAT and SVF isolated from one gram of SAT and VAT (n=4) cultured for 24 hours in the presence or absence of pralnacasan (100μM). C. Secretion of IL-6, IL-8, TNFα and adiponectin by intact VAT (n=5) cultured for 24 hours in the presence or absence of pralnacasan (100μM). * = P-value<0.05, ** P-value<0.01 using a Wilcoxon rank test.

The diminished IL-1β production in VAT was observed in both adipocytes and SVF cells, illustrating that caspase-1 is functionally active in both cellular fractions (Figure 4B). Interestingly, inhibition of caspase-1 activity also limited the boosted production of IL-6 (P-value=0.06) and IL-8 (P-value<0.05) by VAT, yet no effect was observed on adiponectin and TNFα secretion levels (Figure 4C). These results demonstrate that caspase-1 activity is mainly responsible for the production of IL-1β and IL-18 by VAT.

The percentage of CD8$^+$ T-cells in adipose tissue positively correlates with caspase-1 activity levels

Adipose tissue inflammation is partly caused by the influx of immune cells including macrophages, monocytes, and T-cells (30-33). To examine whether the intrinsic activity of caspase-1 in adipose tissue is associated with the immune cell composition, we studied correlations between caspase-1 activity levels and the number of immune cells present in both adipose tissue depots as determined by FACS analysis (Table 1). As shown in figure 5, a significant positive correlation was observed between caspase-1 activity levels and the percentage of CD8$^+$ T-cells present in adipose tissue. Noticeably, none of the other cells measured by FACS analysis (Table 1) correlated significantly with caspase-1 activity levels (data not shown). Interestingly, CD8$^+$ T-cells have been shown to serve as a major contributor to adipose tissue inflammation (34). In line with the differences in caspase-1 activity levels between VAT and SAT, the number of CD8$^+$ T-cells was significantly lower in SAT (Table 1). Moreover, both in SAT and VAT separately, a positive correlation was observed between caspase-1 activity levels and the CD8$^+$ T-cells present in adipose tissue (data not shown). These results suggest that caspase-1 activity in adipose tissue is associated with the influx of CD8$^+$ T-cells.

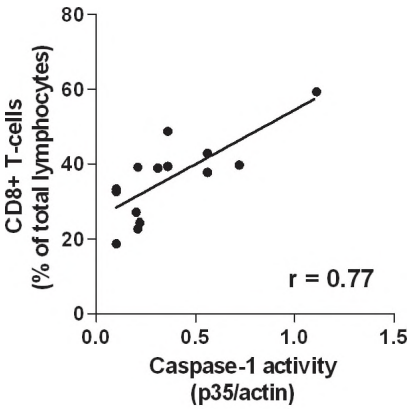

Figure 5. **Caspase-1 activity levels correlates positively with CD8$^+$ T-cell number present in both SAT and VAT.** Caspase-1 activity is represented by the density of the active caspase-1 band (p35). Percentage of CD8$^+$ T-lymphocytes in the SVF from both SAT and VAT were obtained by FACS analysis (n=7) P-value<0.01 using Spearman's rank correlation.

Discussion

In this descriptive study, we demonstrate that in paired human adipose tissue biopsies the innate immune system represented by the NLRP3 inflammasome is abundantly present in VAT compared to SAT. In addition, intrinsic caspase-1 activity is elevated in VAT contributing to the production of IL-1β and IL-18 as well as IL-6 and IL-8. Moreover, caspase-1 activity levels positively correlated with $CD8^+$ T-cells present in the adipose tissue.

In addition to the storage of excessive amounts of energy, adipose tissue has been identified as a source of many inflammatory mediators (1). Interestingly, obesity-induced low-grade inflammation originating from expanding adipose tissue, exploits similar pathways initiated by host defense mechanisms suggestive of an important function of the innate immune system in fat (5;35). Circulating levels of IL-1β and IL-18, both part of the innate immune response, are increased in obese and insulin resistant individuals and have robust effects on atherosclerosis and insulin resistance (36;37). Several reports have identified adipose tissue as a potent source of IL-18 and IL-1β (4;23;38). Human adipose tissue depots have unique inflammatory characteristics exemplified by enhanced production of IL-6, IL-8, TNF-α, and CRP by VAT compared to SAT, that contribute to key features of the metabolic syndrome (7;8;39-41). In line with these studies, we showed an increased production of IL-6, IL-8 and IL-1Ra, together with lower secretion levels of adiponectin in VAT explants.
In this study, we extended the pro-inflammatory properties of VAT by demonstrating that the protein levels of the inflammasome members NLRP3 and ASC were more expressed in this specific depot and that caspase-1 activation is severely increased in VAT compared to SAT resulting in a higher production of IL-1β and IL-18. In addition, secretion levels of the anti-inflammatory cytokine IL-1Ra by VAT were also enhanced and may be the result of a compensatory protective response aimed at counteracting the excessive IL-1β secretion by VAT. Caspase-1 dependent production of IL-1β and IL-18 is supported by the observation that blocking caspase-1 activity in VAT by pralnacasan reduces the secretion of both cytokines. Furthermore, IL-1β release was reduced in both adipocytes and SVF after inhibiting caspase-1, indicating that this enzyme is involved in the IL-1β production in both fractions of VAT.
The caspase-1 dependent release of IL-6 and IL-8 by VAT fits with the well-known capacity of IL-1β to enhance the production of IL-6 and IL-8 by adipose tissue (42;43). Our results show that activation of caspase-1 controls the production of these pro-inflammatory proteins by VAT. However, secretion levels of TNFα from both fat depots were comparable suggesting that the enhanced release of IL-1β and IL-18 is conveyed by a specific mechanism and does not involve a general increase in inflammatory status of VAT.

Although our study clearly demonstrated that IL-1β production was mainly derived from the SVF within VAT, mature adipocytes were also capable to release IL-1β. We hypothesize that in-vivo, an interaction between adipocytes and various cells from the SVF determine the caspase-1 dependent cytokine-secreting capacity of the adipose tissue, hence explaining the high levels of caspase-1 gene expression in adipocytes. Additionally, these high mRNA expression levels in adipocytes may also indicate IL-1β independent effects. Caspase-1 has been shown to regulate insulin sensitivity and to suppress PPAR-γ activity in adipocytes (23;44). Despite the enhanced IL-1β production in VAT compared to SAT and elevated protein levels of caspase-1 and ASC analyzed by western blot, we would like to emphasize that these outcomes can only be used as indirect measurements of inflammasome-dependent caspase-1 activation since the inflammasome depends on protein-protein interactions to activate caspase-1.

Even though it has been clearly established that VAT displays enhanced inflammatory properties compared to SAT, much less is known about the underlying molecular mechanisms. Macrophage infiltration is known to contribute to the inflammatory status of a tissue (28). In the present study, FACS analysis revealed no differences in macrophage content in both fat depots, although we did not differentiate between the resident macrophage populations and the infiltrating macrophages that may represent the primary pro-inflammatory cells (45). The percentage of adipose tissue macrophages in this study was relatively low compared subjects suffering from severe obesity (46). This could be explained by inclusion of solely (healthy) overweight subjects (average BMI: 26.1 kg/m^2 ± SD 2.7). In addition to macrophages, the percentage of other immune cells that are part of the innate immune system (monocytes and granulocytes) present in the SVF of VAT and SAT did not differ. These results rule out differences in caspase-1 activity in VAT compared to SAT due to the influx of innate immune cells. However, in this study caspase-1 activity was associated with the infiltration of cytotoxic T-lymphocytes into adipose tissue. A robust positive correlation was observed between caspase-1 activity levels and the number of CD8$^+$ T-cells present in adipose tissue. Moreover, differences in the percentage of CD8$^+$ T-cells in VAT compared to SAT were mirrored by similar changes in caspase-1 activity levels. Although we did not study the direct effect of caspase-1 on CD8$^+$ T-cell influx in the adipose tissue, caspase-1 itself or by its activation of IL-1β and IL-18 might control the activation and number of CD8$^+$ T-cells present in human adipose tissue. Inasmuch a recent study has demonstrated an important role for CD8$^+$ T-cells in determining adipose tissue inflammation, the influx of these T-cells may represent an important mechanism by which caspase-1 controls adipose tissue inflammation (34). However, further studies will be needed to reveal the possible role of caspase-1 in controlling the influx of CD8$^+$ T-cells into adipose tissue.

Future research should be aimed at identifying possible signals that trigger caspase-1 activation specifically in VAT. The enhanced rate of lipolysis and resistance to insulin action in VAT (47;48) may contribute to elevated activity levels of caspase-1. Hyperglycemia may also be one of the stimulators of caspase-1 in VAT (19), although it remains to be determined why high glucose levels would specifically activate caspase-1 in VAT and not in SAT. In addition to NLRP3, other members of the NLR family that can activate caspase-1, including NLRP1, NLRC4 (IPAF), should be studied in human adipose tissue.

Irrespective of the cellular origin or activators of caspase-1, we demonstrate that the differences in IL-1β and IL-18 release in VAT are mediated by caspase-1. Previously, it has been suggested that expression and regulation of IL-1β are not solely dependent on inflammasome-mediated caspase-1 processing (49;50). Several studies have identified other enzymes that can process pro-IL-1β and pro-IL-18 into their active forms including the neutrophil-derived proteinase-3 under circumstances when neutrophils infiltrate sites of infection (51). Interestingly, it has been reported that neutrophils are activated to a greater extend in obese subjects (52), suggesting that these cells may also contribute to processing of bioactive IL-1β and IL-18. However, FACS analysis revealed no difference in granulocyte infiltration in both fat depots, making it less likely that IL-1β and IL-18 production by VAT occurred independently of caspase-1.

Whereas our study population was composed of a relatively small number of overweight subjects, future studies should be aimed at comparing caspase-1 levels in severely obese and non-obese individuals with or without type 2 diabetes mellitus in larger study populations. Hypothetically, enhanced caspase-1 activation in VAT of severely obese individuals may explain why an increase in this fat depot is associated with an enhanced pro-inflammatory status that may contribute to the progression of cardiovascular disease and type 2 diabetes mellitus.

In conclusion, we demonstrate that the inflammasome components NLRP3, ASC and caspase-1 are present in human abdominal adipose tissue and are highly activated in VAT compared to SAT. Caspase-1 activation leads to an increased release of IL-1β and IL-18, regulates the production of other pro-inflammatory cytokines including IL-6 and IL-8 and appears to be associated with the number of $CD8^+$ T-cells present in adipose tissue. These findings give new insight into the important function of caspase-1 activation in determining the inflammatory characteristics of human adipose tissue.

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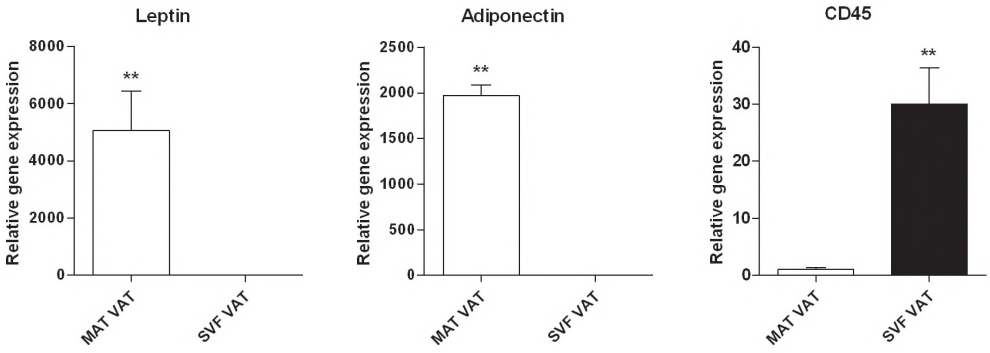
Supplemental data

Supplemental table 1

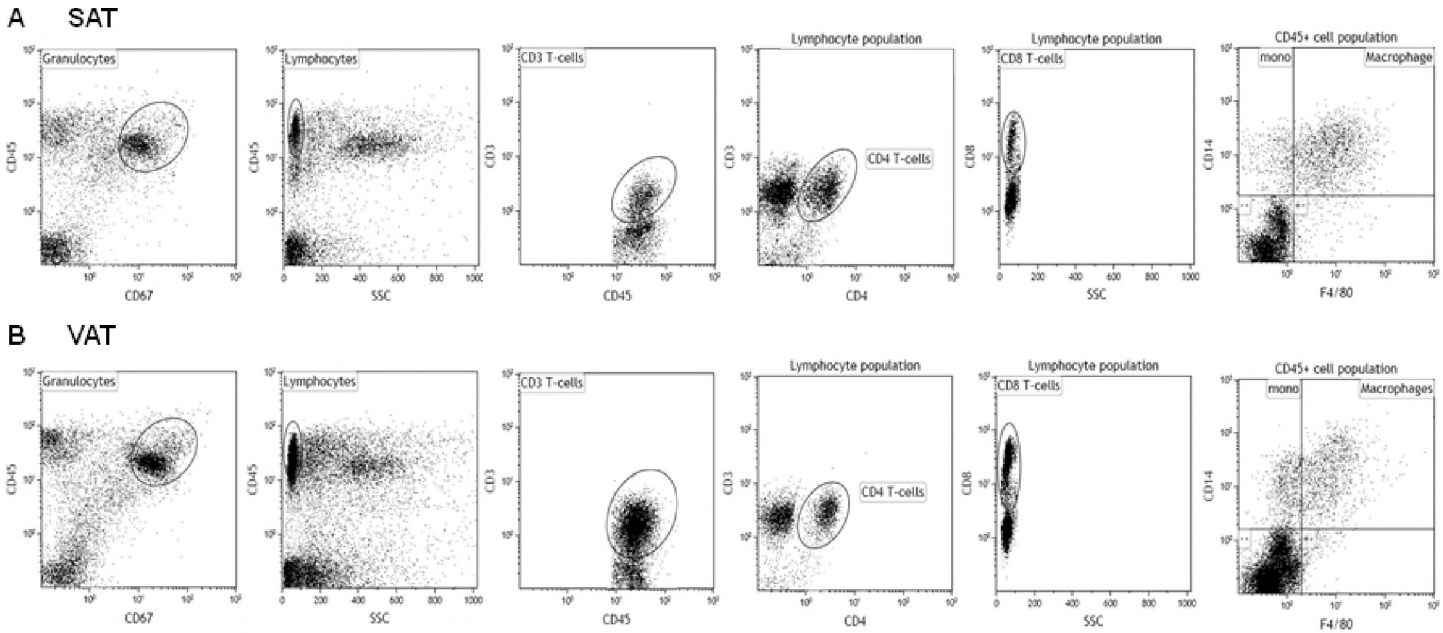
Characteristics of the study population

Characteristics	All
N (male/female)	10 (5/5)
Age (years)	50.5 (8.33)
BMI (kg/m^2)	26.1 (2.73)
WHR	0.90 (0.06)
Glucose (mmol/L)	5.1 (0.65)
hsCRP (mg/L)	0.29 (0.24)

Data are presented as means ± SD. BMI, body mass index; WHR, waist-to-hip ratio; Glucose; fasting levels of plasma glucose hsCRP, plasma levels of high sensitivity C-reactive protein.



Supplementary figure 1. **Purity of adipose tissue fractioning.** Analysis of adiponectin, leptin, and CD45 gene expression levels in the SVF and MAT fraction from VAT. ** = *P*-value<0.01 using a Student's T-test.



Supplementary figure 2. **Immune cell composition of the SVF of human SAT and VAT.** Representative dot plots of flow cytometry data showing immune cells that are part of the innate immune system (granulocytes ($CD45^+$; $CD67^+$), monocytes ($CD45^+$; $CD14^+$; $F4/80^-$) and macrophages ($CD45^+$; $CD14^+$; $F4/80^+$)) and adaptive immune system (total lymphocytes ($CD45^+$ cells plotted against side-scatter area), $CD4^+$ T-cells ($CD3^+$; $CD4^+$ within the lymphocyte population) and $CD8^+$ T-cells ($CD8^+$ plotted against side-scatter area within the lymphocyte population)) within the SVF cells of SAT (A) and VAT (B).

Chapter 8:

General discussion and future perspectives

Inflammatory Adipose (T)issue: Central Role for IL-1β

Obesity-induced insulin resistance is associated with elevated serum levels of pro-inflammatory mediators including the IL-1 family members IL-1β and IL-18 (1-3). Despite a lot of research during the past years, the underlying mechanism which trigger the development of insulin resistance and type 2 diabetes mellitus remain largely unknown. However, results of numerous animal studies, *in-vitro* experiments and clinical trials have generated evidence that IL-1β-driven inflammation in pancreatic beta-cells plays an important role in the pathogenesis of type 2 diabetes mellitus (2;4-6). Additionally, inflammation originating from adipose tissue has been shown to drive the development of metabolic abnormalities including insulin resistance (7;8). However, the central role of the IL-1β-driven inflammatory response in adipose tissue has not been clearly described.

Inflammation is a physiological mechanism necessary to defend the host from external and internal "danger" signals, such as pathogens, DNA damage and metabolic stress (9). Continuous presence of these "danger" signals, which may occur during the development of obesity, could eventually result in an immunogenic reaction in adipose tissue. The inflammatory response in adipose tissue which promotes the development of insulin resistance needs several components that include: 1) a trigger to induce an inflammatory response, 2) a sensor recognizing the trigger, 3) inflammatory mediators that regulate the immune response and 4) influx of immune cells which contribute to the inflammatory response.

Deciphering the inflammatory cascade in obesity-induced insulin resistance will ultimately lead to more effective and specific anti-inflammatory interventions. Based on these four inflammatory components, we will postulate a concept in which the inflammasome-mediated caspase-1 activation plays an important role in controlling adipose tissue inflammation during the development of obesity and insulin resistance through its control of IL-1β release. This results in a vicious circle of pro-inflammatory mediators secreted by dysfunctional adipose tissue preserving the chronic inflammatory environment, which could eventually lead to insulin resistance.

Metabolic signals triggering adipose tissue inflammation

Several metabolic triggers including excessive amounts of nutrients, have been found to initiate and maintain obesity-induced inflammation (10-12). Hyperglycemia has been linked to a pro-inflammatory environment in different tissues including blood vessels, pancreas and adipose tissue. High levels of glucose increase the ability of circulating monocytes for endothelial cell attachment and induce the expression of IL-1β, IL-6, IL-8 and MCP-1 in adipocytes, endothelial cells and pancreatic beta-cells (13-18). Similar to glucose, elevated levels of free fatty acids (FFAs) induce inflam-

matory cytokine production including IL-1β, IL-6 and TNF-α in pancreatic islets, macrophages and adipocytes (19-21).
Research using pancreatic beta-cells and macrophages gave important clues about which nutrient-derived triggers are involved in the secretion of IL-1β. Nutrient-derived triggers including elevated levels of glucose, FFAs, leptin, oxidized low density lipoprotein (oxLDL), gut microbiota-derived lipoploysaccharide (LPS), cholesterol crystals, and islet amyloid polypeptide have been found to induce the release of bioactive IL-1β (19;22-28). In addition, IL-1β itself is able to increase the levels of pro-IL-1β in an autocrine fashion (5). Our results demonstrated that elevated levels of glucose also increase the secretion of IL-1β in adipose tissue **(chapter 6)**.
Toxicity by elevated levels of glucose and FFAs is mediated, at least to a significant extend, by an increased production of reactive oxygen species (ROS) which lead to oxidative stress in pancreatic beta-cells, macrophages and adipose tissue (16;18;29). Recent evidence suggest that ROS stimulate the secretion of IL-1β in pancreatic beta-cells and macrophages (30). Thus ROS may also play an important role in adipose tissue inflammation during obesity. However, more research is necessary to unravel the involvement of ROS in adipose tissue release of IL-1β upon stimulation with high levels of glucose or free fatty acids.

Cellular sensor mechanism recognizing metabolic triggers: Role of the inflammasome

How does adipose tissue sense these triggers which arise during the development of obesity? Recently, a mechanism has been uncovered in pancreatic beta-cells and macrophages, which involves NLRP3 inflammasome-mediated caspase-1 activation upon stimulation with nutrition-derived ligands (31;32). These results suggest that NLRP3 inflammasome-dependent release of IL-1β contributes to the pathogenesis of type 2 diabetes mellitus. We demonstrated for the first time the presence of NLRP3 inflammasome components in adipose tissue, together with increased levels of caspase-1 activity and IL-1β in adipose tissue of obese mice models, which supports the concept that a similar sensing mechanism exists in adipose tissue **(chapter 5 and 6)**. Indeed, caspase-1-/- mice show a decrease in adipose tissue-derived IL-1β and reveal an improvement of insulin sensitivity as compared to wild-type mice. Thus, caspase-1 is an important link between obesity-induced inflammation in adipose tissue mediated by IL-1β and the development of insulin resistance. Interestingly, we showed that the intrinsic levels of the NLRP3 inflammasome components are significantly higher expressed in visceral adipose tissue (VAT) compared to subcutaneous adipose tissue (SAT), which corresponds with the elevated basal IL-1β production in this specific depot **(chapter 7)**.
As previously mentioned, NLRP3 inflammasome serves as an intracellular sensor

of metabolic stress in mouse pancreatic beta-cells and macrophages resulting in an enhanced release of IL-1β. A crucial role for ROS as a potential activator for NLRP3 has been described (33). The mechanism whereby ROS lead to NLRP3 inflammasome activation upon stimulation with high concentrations of glucose in mouse pancreatic beta-cells has been described by Zhou et al (34). In this model, thioredoxin interacting protein (TXNIP) has a prominent role since it dissociates from the ROS scavenger thioredoxin after oxidative stress and activates the inflammasome by binding NLRP3. Interestingly, TXNIP is also involved in glucose uptake in adipocytes (35). We showed for the first time that TXNIP regulates high glucose-induced IL-1β transcription in adipocytes, without affecting caspase-1 activation (**chapter 6**). Additionally, hyperglycemia tended to elevate protein levels of NLRP3 in adipocytes (**chapter 6**), which fits with the concept that NLRP3 inflammasome-dependent IL-1β release could also be controlled by the expression level of NLRP3 itself (36). All together, these findings indicate that in adipose tissue NLRP3 inflammasome could indeed act as an intracellular sensor detecting nutrient-derived triggers like elevated levels of glucose. However, the pathway which leads to hyperglycemia-induced IL-1β secretion in adipocytes seems to differ from what is observed in pancreatic beta-cells. In contrast to the study of Zhou et al, we did not detect a direct role for TXNIP in caspase-1 activation.

Role of IL-1 family members in adipose tissue inflammation

The IL-1 family members IL-1β and IL-18 are strongly associated with inflammation and partially secreted by adipose tissue from subjects with obesity and insulin resistance (37-39). Whereas increased levels of IL-β are linked to the development of insulin resistance (2;40;41), IL-18 appears to suppress insulin resistance as a consequence of reducing obesity caused by inhibition of food and energy intake, as observed in IL-18 -/- mice (42;43) (**chapter 4**). Paradoxically, obese subjects are often characterized by increased levels of circulating IL-18. This observation may be explained by the development of a lower cellular response to IL-18 in obese subjects, which is subsequently compensated by a higher production of IL-18 similar to hyperinsulinemia and hyperleptinemia (44). These data suggest that IL-1β and IL-18 oppositely affect systemic insulin sensitivity. In chapter 5 we showed that the absence of caspase-1, has a beneficial effect on insulin sensitivity. This suggests that the absence of the adverse effects of IL-1β dominate the increased insulin resistance associated with the lack of IL-18.

In this thesis we demonstrated the central role of IL-1β in adipose tissue inflammation in different ways. First, IL-1β secretion by adipose tissue is higher in animal models associated with obesity and insulin resistance (**chapter 5**). Secondly, hyperglycemia induce the release of IL-1β in adipocytes (**chapter 6**) and finally, VAT secretes higher

levels of IL-1β compared to SAT (**chapter 7**). The hazardous effects of IL-1β in adipose tissue which contribute to the pathophysiology of insulin resistance have been partially unravelled in different human and rodent studies. Elevated levels of IL-1β reduce adipocyte differentiation, stimulate lipolysis and inhibit the insulin signalling pathway in adipocytes (40;45-47) (**chapter 5**).
Impaired adipocyte differentiation could indirectly lead to hypertrophy of the remaining adipocytes. Besides a decrease in adiponectin production, enlarged adipocytes secrete higher levels of the pro-inflammatory mediators leptin, MCP-1, TNF-α, IL-6 and FFAs which promote insulin resistance (48;49). This could induce the infiltration of macrophages into adipose tissue, which contribute to the inflammatory response by inducing the secretion of pro-inflammatory cytokines. Indeed, the number of macrophages in adipose tissue have been shown to correlate directly with adipocyte size (50). Decreased levels of adiponectin are associated with enhanced expression of pro-inflammatory genes including hsCRP, TNF-α and IL-6 (51-53), whereas the production of the anti-inflammatory cytokines IL-1Ra and IL-10 are reduced in macrophages (54;55).
Interestingly, in pancreatic islets IL-1β is capable to potentiate its own secretion by auto-stimulation mediated by NF-κB, which is a known activator of the IL-1β promoter (5;56). This auto-stimulation process has not been described in adipose tissue so far. However, it is possible that this mechanism is present in adipose tissue amplifying the immune response, yet more research is needed to verify this hypothesis. In addition, IL-1β is a potent inducer of IL-6 and IL-8 in adipose tissue and blocking the effects of endogenous IL-1β in an adipose tissue explant culture with a neutralizing antibody results in a decrease of both IL-6 and IL-8 (57).
All together, IL-1β-induced inflammation creates a disturbed balance between pro-inflammatory and anti-inflammatory mediators in adipose tissue, which may lead to an increased risk to develop type 2 diabetes and CVD.

Cells contributing to the IL-1β-mediated inflammatory response within the adipose tissue

Initially, it was thought that IL-1β was primarily produced by cells of the innate immune system (58;59). However, it became clear that cells outside the immune system secrete IL-1β including pancreatic beta-cells and adipocytes (15;60). Adipose tissue of subjects suffering from obesity and insulin resistance is characterized by an influx of macrophages (50;61). Together, these cells may contribute to increased IL-1β production due to coherence and create an inflammatory environment in the adipose tissue. This is in line with the IL-1β transcription levels observed in the different cellular fractions of adipose tissue. Although IL-1β is expressed by isolated adipocytes, IL-1β transcription levels are significantly higher in stromal vascular fraction (SVF)

derived from adipose tissue (**chapter 7**). Comparison of the expression levels of the inflammasome members in the different adipose tissue fractions revealed that NLRP3 is equally distributed among the different adipose tissue fractions, caspase-1 is significantly elevated in mature adipocytes, whereas the expression of adapter protein apoptosis-associated specklike protein (ASC) is higher in the SVF fraction. This discrepancy in inflammasome expression could suggest a crosstalk between adipocytes and immune cells in the adipose tissue regulating and amplifying IL-1β production. However, additional studies are needed to single out the contribution of each adipose tissue fraction to produce IL-1β.
Interestingly, we reported a strong positive correlation between caspase-1 activation and the presence of $CD8^+$ T-lymphocytes (cytotoxic T-cells) in both VAT and SAT of overweight subjects (**chapter 7**). The contribution of this pro-inflammatory T-cell subset in adipose tissue inflammation has been revealed in a study by Nishimura et al., who described that cytotoxic T-cells precede the invasion of macrophages into adipose tissue during the onset of obesity (62). Although more studies will be needed to determine the exact underlying cause of the association between caspase-1 and cytotoxic T-cells, caspase-1 itself or via IL-1β activation may control the number of cytotoxic T-cells present in human adipose tissue. This concept is supported by a study demonstrating that IL-1β induces the secretion of the chemokine IP-10 in mature adipocytes which regulates the migration of T-cells into adipose tissue (63).
All together, we demonstrated that both the adipocyte fraction and the immune cells in the SVF fraction may contribute to the caspase-1-dependent production of IL-1β. Furthermore, increased caspase-1 activation in adipose tissue is associated with an increased number of cytotoxic T-cells, which may contribute to the progression of the inflammatory response.

Therapeutic and lifestyle interventions to inhibit obesity-induced inflammation

Normally, the acute immune response is terminated when the initial trigger has been eliminated. However, during obesity, adipose tissue is continuously exposed to nutrient-derived triggers, which results in persistent low-grade inflammation. Suppressing the inflammatory response by modulating one of the four critical components of the inflammatory cascade within adipose tissue inflammation, will lead to beneficial effects on insulin sensitivity. First of all, there have been some experiments with ROS inhibitors which target oxidative stress in cells resulting in a restoration of insulin sensitivity (18;64). This might also be a promising intervention in reducing adipose tissue inflammation. Other targets that could suppress the inflammatory response in adipose tissue include inhibitors of the inflammasome sensing mechanism. In line with this, we clearly demonstrated that treatment of Ob/Ob mice, characterized by

insulin resistance, display a reduction in weight gain and improvement of insulin sensitivity after oral treatment with a caspase-1 inhibitor. This could be explained by the loss of the direct deleterious metabolic effects of IL-1β on adipogenesis and improved adipose tissue insulin sensitivity. Additionally, metabolic cage analysis reveals that caspase-1-/- animals are characterized by an enhancement in fat oxidation rate, which may contribute to the beneficial effects on adiposity compared to wild-type animals (**chapter 5**). Recent data have also revealed that the absence of caspase-1 prevents the influx of macrophages into adipose tissue during high fat diet-feeding (unpublished data). Furthermore, blocking caspase-1 results in a diminished pro-inflammatory secretion profile in VAT by reducing the IL-β-induced production of IL-6 and IL-8. Specifically targeting NLRP3 would also be an interesting probability to suppress the release of IL-1β, since this inflammasome component has mainly been ascribed to sense metabolic abnormalities associated with obesity. Moreover, inflammasomes consisting of other cytoplasmic sensors would not be affected in this way, which prevents the lack of a decent immune response during infection.

Until now, several animal experiments and clinical trials have been performed to study the effects of IL-1β neutralization (antibody against IL-1β) or IL-1 receptor blockade (IL-1Ra treatment) on glycemic control. Blocking the effects of IL-1β in patients with type 2 diabetes mellitus and in insulin resistant animal models, revealed an improvement of glycemic control and pancreatic beta-cell function (4;65). Until now, the beneficial effects of these treatments have not been studied in adipose tissue, yet *in-vitro* experiments with VAT explants suggests that blocking endogenous IL-1β results in a decreased production of pro-inflammatory cytokines (57). Furthermore, blocking IL-1β release from adipocytes prevents hepatic insulin resistance, which suggest that IL-1β could mediate a specific cross talk between adipose tissue and liver (41).

Another approach to disrupt the pro-inflammatory status of adipose tissue, is to reduce adipocyte hypertrophy by treatment with thiazolidinediones (TZDs) or to lose weight. Indeed, a decrease in adipocyte cell surface observed after weight loss and treatment with TZDs normalizes the release of FFAs and TNF-α, which ameliorating insulin sensitivity (66;67). TZDs also increase the expression of adiponectin and stimulate visceral adipocyte differentiation promoting the development of small insulin sensitive adipocytes (68;69). Interestingly, we found that the TZD pioglitazone results in subcutaneous adipocyte enlargement with a reduction in inflammation and improvement of insulin sensitivity (**chapter 3**). This could be explained by redistribution of visceral fat storage towards the SAT depot, leading to smaller, more insulin sensitive visceral adipocytes. Furthermore, it has been found that inducing macrophage-specific PPAR-γ activity promotes differentiation towards macrophages with anti-inflammatory characteristics (70;71). Interestingly, we observed that the TZD rosiglitazone inhibits high glucose-induced IL-1β possibly by down-regulating TXNIP in adipocytes (**chapter 5**). These findings result in a reduction of pro-inflammatory mediators in the

adipose tissue that may contribute to the pathogenesis of insulin resistance.
Other promising research against obesity and metabolic disease has focused on specific nutrients that may have anti-inflammatory potential, the polyunsaturated FFAs. High fat diet-fed mouse treated with additional polyunsaturated FFAs show a decreased expression of pro-inflammatory cytokines, a reduction in macrophage infiltration into adipose tissue and an improvement of systemic insulin sensitivity (72). In contrast to saturated FFAs, polyunsaturated FFAs may exert their anti-inflammatory effects through inhibiting TLR4 signaling, thereby reducing the release of pro-inflammatory mediators (73). In this context it would be interesting to study potential inhibitory effects on inflammasome/caspase-1 activation by polyunsatured FFAs.

Conclusion

In conclusion, elevated levels of glucose accompanied with obesity create an inflammatory environment in adipose tissue by inducing caspase-1-dependent IL-1β production in adipocytes through the NLRP3 inflammasome. During the onset of obesity, IL-1β contributes to the progression of adipocyte hypertrophy by inhibiting differentiation of newly formed adipocytes resulting in adipocyte dysfunction characterized by an increased pro-inflammatory cytokine and chemokine secretion profile. This contributes to the recruitment of immune cells including cytotoxic T-cells and macrophages into the adipose tissue, which further potentiates the inflammatory response. This inflammatory cascade, induced by caspase-1 activation, seems to be more pronounced VAT as compared to SAT which may explain why accumulation of the visceral fat depot contributes to the increased risk to develop insulin resistance or CVD (74). Therefore, we would like to propose a central role for caspase-1-mediated IL-1β activation in adipose tissue inflammation during obesity (Figure 1).

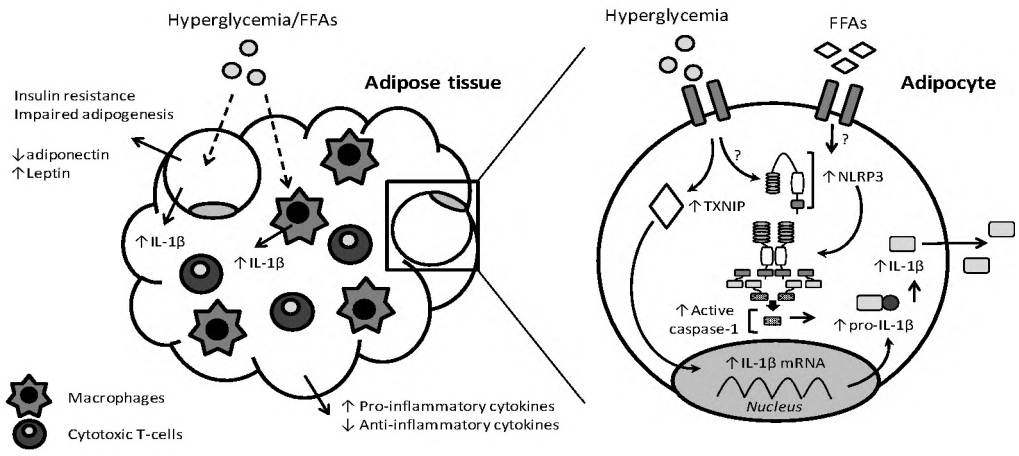


Figure 1. **IL-1β-mediated inflammation in adipose tissue during obesity.** Metabolic triggers like hyperglycemia, and perhaps free fatty acids (FFAs), promote IL-1β in hypertrophic adipocytes by activating two separate pathways. 1). inducing IL-1β mRNA transcription via up-regulating of TXNIP expression 2). increasing (NLRP3 inflammasome-dependent) caspase-1 activation. This leads to the release of active IL-1β by the adipose tissue. IL-1β inhibits adipocyte differentiation and induce expression of pro-inflammatory mediators (IL-6, IL-8 TNF-α, IP-10 and MCP-1), which recruit cytotoxic T-cells and macrophages into the adipose tissue. The immune cells contribute to a broad immune response and infiltrated macrophages further promote the release of IL-1β and other cytokines (IL-18 and TNF-α).

Future perspectives

Since the discovery that obesity-induced insulin resistance is accompanied by a systemic low-grade inflammation originating from adipose tissue, many efforts have been made to unravel the triggers of the inflammatory response and the subsequently activated mechanisms which finally result in the development of systemic insulin resistance. Although our data suggests an important role for caspase-1 in regulating adipose tissue inflammation by mediating IL-1β activity, some unanswered questions still remain.

Like discussed in chapter 8, most of the research that aims at identification of obesity-induced triggers and its sensors has been performed in pancreatic beta-cells and macrophages. Except the role of hyperglycemia studied in this thesis, it is unknown whether triggers like oxidized LDL, FFAs and cholesterol crystals also lead to caspase-1 mediated IL-1β release in adipose tissue.

Apart from the NLRP3 inflammasome, other inflammasomes have been described based upon the NLRs cytoplasmic sensor component including NLRP1 and NLRC4. Recently, the protein absent in melanoma 2 (AIM2) has also been reported to have the capacity to assemble an inflammasome. Therefore, future research should focus on the identification of the specific inflammasomes that may contribute to the obesity-induced adipose tissue inflammation.

In chapter 7 we observed an increase in caspase-1 activation in VAT compared to SAT, which may partly explain the pro-inflammatory character of this fat depot. However, the paired adipose tissue biopsies were obtained from mildly overweight subjects only. Therefore, it would be interesting to study caspase-1 activation in fat depots from both lean and obese subjects or from patients suffering from type 2 diabetes and familial combined hyperlipidemia (FCH). This will give valuable insights into the role of caspase-1-mediated inflammation in SAT and VAT and the pathogenesis of insulin resistance and type 2 diabetes mellitus.

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Chapter 9

Summary and conclusions

Summary

Obesity is characterized by excessive adipose tissue accumulation, caused by an inactive lifestyle, increased food intake and genetic factors that may predispose to excessive weight gain. Obesity is associated with an increased risk to develop metabolic disorders that include insulin resistance, type 2 diabetes mellitus and cardiovascular diseases (CVD). Alterations in the endocrine characteristics of the adipose tissue play a major role in mediating these metabolic abnormalities including a disturbed secretion of various adipokines that leads to changes in energy homeostasis, lipid- and glucose metabolism. These mostly adverse metabolic alterations are associated with profound changes in adipose tissue morphology that include an increased number of large dysfunctional adipocytes.
Research has singled out inflammation as one of the most important links between obesity and the development of metabolic abnormalities. Elevated levels of circulating cytokines are observed in insulin resistant subjects and closely correlate with an increase in adipose tissue mass. More specifically, it has been shown that the enhanced inflammatory status observed in obese subjects primarily originates from expanding visceral adipose tissue (VAT) whereas the enlargement of the subcutaneous adipose tissue (SAT) depot is relatively harmless.
Only since the early 90's, our view of the adipose tissue as solely an organ to store excessive amounts of energy has dramatically changed as several studies revealed that the adipose tissue is a prominent source of pro-inflammatory mediators in obese individuals. TNF-α, MCP-1, IL-6, IL-8, IL-1β and IL-18 are among a rapidly growing list of pro-inflammatory mediators secreted by the adipose tissue during the development of obesity. Most importantly, inflammation of the adipose tissue is accompanied by an influx of a variety of immune cells including macrophages, neutrophils, and T-lymphocytes. These pro-inflammatory characteristics are more closely linked to expanding VAT as compared to SAT. However, the exact mechanisms by which obesity contributes to the development of metabolic abnormalities via propagation of adipose tissue inflammation are still unknown.

Pathogenic role of adipose tissue in metabolic disorders

Disturbed adipose tissue function: altered adiponectin secretion
We demonstrated that total plasma levels of the anti-inflammatory and insulin-sensitizing protein adiponectin were reduced in subjects diagnosed with familial combined hyperlipidemia (FCH), who are characterized by elevated levels of cholesterol, triglycerides, apo-lipoprotein B and insulin resistance. Adiponectin circulates in various multimeric isoforms: low molecular weight (LMW), middle molecular weight (MMW) and high molecular weight (HMW) isoform. Additional studies have shown

that the favourable metabolic and anti-inflammatory effects of adiponectin are mainly mediated by the HMW isoform of adiponectin. Therefore the beneficial circulating adiponectin profile is often expressed as the ratio between HMW and total adiponectin, whereas a more unfavourable adiponectin profile is expressed as the LMW to HMW ratio. Therefore, we investigated whether a reduced plasma level of total adiponectin in subjects with FCH was accompanied by a change in adiponectin multimer distribution. In addition, we studied the association between the circulating concentrations of the different adiponectin multimeric isoforms and the presence of CVD in patients suffering from FCH (**chapter 2**).
The decreased systemic levels of total adiponectin found in subjects with FCH compared to healthy controls was mainly attributed to a reduction in both the LMW and HMW isoforms of adiponectin. However, the ratio between the HMW isoform and total adiponectin and the LMW/HMW adiponectin ratio did not differ between FCH and control subjects. The adiponectin multimer distribution differed significantly between male and female subjects in both study populations and the more favourable distribution was observed in females. Interestingly, within the group of females diagnosed with FCH, the presence of CVD correlated with a reduction of the HMW form of adiponectin compared to women without CVD (HMW/total adiponectin: 34.2 ± 10% vs. 46.0 ± 7.1% for FCH females with and without CVD, respectively; LMW/HMW adiponectin ratio: 1.3 ± 0.8 and 0.9 ± 0.7 for FCH females with and without CVD, respectively). Inasmuch adiponectin has been attributed with anti-inflammatory properties including the reduction of NF-κB activity, the adverse multimer secretion profile originating from adipose tissue might promote a more pro-inflammatory environment that increases the risk to develop CVD.

Adipocyte hypertrophy in relation to adipocyte dysfunction
Obesity is associated with hypertrophic adipocytes, which are characterized by pro-inflammatory properties promoting the infiltration of macrophages into adipose tissue that lead to the development of insulin insensitivity. Thiazolidinediones (TZDs) are an insulin-sensitizing class of pharmaceutical compounds used for the treatment of type 2 diabetes mellitus that directly affect adipose tissue by improvement of adipocyte differentiation and glucose homeostasis through activation of PPARγ. In a study with congenital adrenal hyperplasia (CAH) subjects who suffer from insulin resistance, we investigated the effects of the TZD pioglitazone on insulin sensitivity, subcutaneous adipocyte surface, and adipose tissue-specific gene expression (**chapter 3**).
Before treatment, mean subcutaneous adipocyte surface showed clear inter-individual differences and was positively correlated with the percentage of trunk fat (r=0.73; *P*-value=0.01), plasma leptin (r=0.70; *P*-value=0.01) and MCP-1 gene expression levels in adipose tissue (r=0.57; *P*-value=0.05). Adipocyte cell surface was negatively correlated with systemic insulin sensitivity (r= -0.60; *P*-value=0.04) and adipose tissue expression of the glucose uptake transporter (GLUT)-4 (r=-0.65; *P*-value=0.03).

Treatment with pioglitazone resulted in an improvement of systemic insulin sensitivity, accompanied by an increase in BMI (by 7 ± 1.1 kg/m^2; *P*-value= 0.06) and subcutaneous adipocyte cell surface (by 497 ± 625 μm^2; *P*-value< 0.05). Furthermore, gene expression levels of insulin sensitivity markers adiponectin and GLUT-4 were up-regulated, whereas expression of the pro-inflammatory gene MCP-1 was decreased. Interestingly, the percentage of trunk fat, plasma leptin levels and MCP-1 gene expression were no longer positively correlated with subcutaneous adipocyte surface. This implies that the impaired metabolic characteristics of hypertrophic adipocytes are diminished after treatment with pioglitazone, while the mean subcutaneous adipocyte cell surface was even further increased. We hypothesize that the pioglitazone-induced enlargement of adipocyte cell surface results from redistribution fat from VAT depots towards storage in SAT. This redistribution may alleviate the pro-inflammatory trait of VAT by reducing the number of large adipocytes and enhancing the percentage of insulin sensitive smaller visceral adipocytes.

Inflammasome-mediated caspase-1 activation in adipose tissue

IL-18 protects against the development of obesity-induced insulin resistance

Obesity-induced inflammation and insulin resistance are characterized by elevated circulating cytokine levels. It is generally believed that obesity-induced inflammatory mediators originate from expanding adipose tissue. However, fat may also accumulate in other tissues including the liver during the development of obesity and may promote hepatic inflammation.

Cytokines have divergent effects on insulin sensitivity. While TNF-α, IL-6, IL-8 and IL-1β promote obesity-induced insulin resistance others appear to promote insulin sensitivity. Plasma levels of the prominent IL-1 family members IL-1β and IL-18 are increased during obesity and associated with insulin resistance. However, IL-18 has been shown to improve insulin sensitivity and prevent the development of obesity partly by controlling food intake, yet the underlying molecular mechanisms remain unknown. Therefore, we set out to determine the contribution of IL-18 to the development of obesity-induced inflammation and insulin resistance by feeding both wild-type and IL-18-/- animals a high fat diet (HFD) to promote obesity (**chapter 4**). To our surprise, HFD-induced weight gain was similar in both IL-18-/- and wild-type animals. However, IL-18 knockout animals were more insulin resistant compared to wild-type mice fed the HFD. Furthermore, HFD-fed IL-18-/- mice displayed metabolic abnormalities including higher levels of circulating VLDL triglycerides and lower plasma adiponectin levels compared to wild-type animals fed the HFD. Since IL-18 is highly expressed in adipose tissue and liver, it suggests that these tissues play a prominent role in translating the insulin-sensitizing effects of IL-18. Interestingly, treatment of IL-18-/- mice with recombinant IL-18 resulted in an improvement

of hepatic insulin sensitivity. Detailed analysis of the liver-specific effects of IL-18 during the development of obesity revealed no difference in the degree of hepatic steatosis between both genotypes fed the HFD. However, plasma ALT levels were clearly elevated in IL-18-/- fed the HFD compared to the wild-type controls, suggestive of liver damage. Adipose tissue morphology did not differ between wild-type and IL-18-/- animals. Surprisingly, influx of macrophages into adipose tissue revealed a reduction in HFD-fed IL-18-/- animals in conjunction with lower levels of MCP-1, yet gene expression levels in adipose tissue of the pro-inflammatory cytokine IL-6 were increased, whereas levels of anti-inflammatory cytokine IL-1Ra were decreased. All together, these findings imply that IL-18 controls insulin sensitivity independently of hepatic steatosis and adipose tissue macrophage influx. IL-18 seems to directly improve hepatic insulin sensitivity, and a lack of IL-18 is accompanied by lower adiponectin secretion levels that may promote insulin resistance.

Inflammasome/caspase-1 controlling adipose tissue insulin sensitivity

In order to become active, the precursors of IL-1β and IL-18 are processed into their active forms by a cysteine protease called caspase-1. Activation of caspase-1 itself involves cleavage by an intracellular protein complex called the inflammasome, consisting of the (NOD)-like receptor (NLR) family member NLRP3 and adapter protein apoptosis-associated specklike protein (ASC). Because IL-1β and IL-18 influence metabolic homeostasis and due to the essential role of inflammasome-mediated caspase-1 to activate these two cytokines, we determined the role of caspase-1 in adipose tissue function (**chapter 5**). Obese insulin resistant mouse models were characterized by an increased activation of caspase-1 in adipose tissue together with elevated levels of IL-1β and IL-18 in adipose tissue. Depletion of caspase-1 in adipocytes improved adipogenesis (increased gene expression of PPAR-γ, adiponectin, GLUT-4) and insulin sensitivity as determined by an increased phosphorylation of AKT (mock vs. caspase-1 siRNA, 0.95 ± 0.14 vs. 2.10 ± 0.16; *P*-value<0.005). These effects of caspase-1 were likely conveyed by processing of IL-1β. Whereas treatment of adipocytes with an anti-IL-1β antibody mirrored the effects of caspase-1 inhibition, recombinant IL-18 treatment had no effect on adipogenesis. Absence of NLRP3 mimics the effects seen in caspase-1-/- animals, with an enhanced adipocyte differentiation and improved adipose tissue insulin sensitivity. In line with the increased adipose tissue-specific insulin sensitivity, IL-1β release from adipose tissue was reduced in caspase-1 and NLRP3 deficient animals. *In-vivo* experiments with caspase-1-/- animals revealed an improvement of systemic insulin sensitivity compared to wild-type littermates. In parallel, treatment of Ob/Ob mice, characterized by an excessive bodyweight and the presence of insulin resistance, with a caspase-1 inhibitor improved insulin sensitivity and led to a reduction in bodyweight gain. The absence of caspase-1 led to a reduction in fat mass and smaller adipocytes (mean adipocyte cell surface, wild-type mice 966.8 μM^2, caspase-1-/- mice 629.16 μM^2; *P*-value<0.001). Furthermore, indirect calorimet-

ric analysis revealed higher fat oxidation rates in caspase-1-/- animals. These results demonstrated that inflammasome-mediated caspase-1 activation in adipose tissue during obesity leads to increased IL-1β production and contributes to the induction of tissue-specific and systemic insulin resistance, whereas the absence or inhibition of caspase-1 improved insulin sensitivity and adipose tissue function. These results imply that pharmaceutical inhibition of caspase-1 could be a potential therapeutic target in the treatment of obesity and type 2 diabetes mellitus.

Hyperglycemia-mediated caspase-1 activation and IL-1β secretion in adipose tissue

The importance of IL-1β in obesity-induced inflammation and the development of insulin resistance in adipose tissue have been well documented in several studies. In addition, we demonstrated that inflammasome/caspase-1 affects adipose tissue function during the development of obesity (**chapter 5**), yet the exact triggers leading to caspase-1 activation in adipose tissue remain unknown. Interestingly, a recent study demonstrated that hyperglycemia induces an interaction between thioredoxin interacting protein (TXNIP) and NLRP3 that subsequently leads to caspase-1 activation and IL-1β production by mouse pancreatic beta-cells. We determined whether a similar mechanism may exist in human adipocytes and intact adipose tissue (**chapter 6**). Treatment of human intact adipose tissue and primary adipocytes with high levels of glucose led to an increase in IL-1β transcription levels (7.5-fold; P-value<0.01, 1.7-fold; P-value<0.05, respectively), intracellular protein levels of pro-IL-1β (2.0-fold; P-value<0.01, 1.5-fold; P-value<0.05, respectively) and secretion of bioactive IL-1 (2.0-fold; P-value<0.01, 3.0-fold; P-value<0.05, respectively). Additionally, caspase-1 activity levels were elevated by 10% (P-value<0.05) and NLRP3 protein levels were increased in human adipocytes treated with high levels of glucose. In addition to caspase-1 and NLRP3, TXNIP protein levels increased in response to elevated levels of glucose in both human adipocytes and intact adipose tissue (2-fold; P-value<0.05, 5-fold; P-value<0.01, respectively). High glucose-induced bioactive IL-1 secretion was reduced in adipocytes targeted with siRNA against TXNIP. This was accompanied by a significant 2-fold decrease in IL-1β transcription levels and intracellular levels of pro-IL-1β. However, depletion of TXNIP had no effect on caspase-1 activation when adipocytes were stimulated with high levels of glucose.

These results demonstrated that hyperglycemia could serve as a trigger for NLRP3 inflammasome mediated-caspase-1 activation. Furthermore, elevated levels of glucose induced adipocyte-specific TXNIP expression that subsequently led to increased IL-1β transcription. However, in adipose tissue TXNIP did not appear to directly stimulate caspase-1 activation. In conclusion, hyperglycemia independently induces both TXNIP-mediated IL-1β transcription and caspase-1 activation that together lead to enhanced production of IL-1β that may promote insulin resistance.

Inflammasome and caspase-1 activation in VAT

It has been suggested that VAT, rather than SAT, contributes to the elevated circulating levels of pro-inflammatory cytokines in obese subjects. The enhanced inflammatory properties of VAT compared to SAT may be attributed to an augmented influx of different immune cells that promote the production of a variety of inflammatory mediators. Although the inflammasome-mediated IL-1β and IL-18 have also been linked to obesity and insulin resistance and they partly originate from adipose tissue, it is currently unknown whether inflammasome/caspase-1 activity contributes to the enhanced pro-inflammatory status of VAT versus SAT. Therefore, we set out to study the expression profile of the NLRP3 inflammasome components in paired VAT and SAT biopsies from overweight subjects both by using mRNA expression and protein analysis (**chapter 7**). In this study, the cellular composition of the stromal vascular fraction of VAT was characterized by an increased number of $CD8^+$ T-lymphocytes compared to SAT (30.4% vs. 41.6%; *P*-value<0.05). No differences in macrophage number or in adipocyte cell size surface were observed between VAT and SAT. However, *ex-vivo* adipose tissue cultures demonstrated enhanced intrinsic inflammatory properties of VAT illustrated by a boosted release of IL-6, IL-8, IL-1Ra (3-fold; *P*-value<0.05, 4-fold; *P*-value<0.05, and 2-fold; *P*-value<0.05, respectively) and decrease in adiponectin secretion levels. Importantly, total IL-1β (pro-form of IL-1β + active form of IL-1β), bioactive IL-1 (10-fold; *P*-value<0.05) and IL-18 (3-fold; *P*-value<0.05) secretion from VAT were enhanced as compared to SAT.

In line with the elevated secretion levels of IL-1β and IL-18, VAT showed a 3-fold (*P*-value<0.05) increase in caspase-1 activation compared to SAT, together with an up regulation of the inflammasome components NLRP3 (2-fold; ns) and ASC (2-fold; *P*-value<0.05) protein levels. The caspase-1 dependent cytokine production by human white adipose tissue was further supported by treatment of VAT explants with pralnacasan, a specific inhibitor of caspase-1, which led to a reduced release of IL-1β and IL-18, yet also diminished the production of IL-6 and IL-8. However, TNFα secretion levels were unaffected by caspase-1 inhibition. Finally, a significant positive correlation was observed between caspase-1 activity levels and the percentage of $CD8^+$ T-lymphocytes present in adipose tissue (r=0.77; *P*-value<0.01). These results suggest that caspase-1 serves as an important contributor to the intrinsic pro-inflammatory trait of VAT via increased production of IL-1β and IL-18, but also by regulating the secretion of IL-6 and IL-8. Furthermore, caspase-1 activity was positively associated with the number of $CD8^+$ T-lymphocytes present in adipose tissue, which are known to contribute to adipose tissue inflammation. Controlling the influx of $CD8^+$ T-lymphocytes may be an additional pathway by which caspase-1 governs adipose tissue inflammation.

Conclusions

1. Reduced circulating levels of total adiponectin in subjects with FCH are not associated with a disturbed adiponectin multimer profile. However, in females diagnosed with FCH, the presence of CVD is accompanied by a more atherogenic adiponectin multimer secretion profile originating from adipose tissue.
2. Enlarged subcutaneous adipocyte cell surface is associated with inflammation and insulin resistance. Pioglitazone treatment uncouples this association illustrated by subcutaneous adipocyte surface enlargement together with a reduction in inflammation and an improvement of systemic insulin sensitivity.
3. The IL-1 family member IL-18 regulates insulin sensitivity independently of the development of obesity and without affecting hepatic steatosis and macrophage infiltration into adipose tissue during high fat diet feeding.
4. Increased inflammasome-mediated caspase-1 activation in adipose tissue during obesity leads to elevated IL-1β production and contributes to the induction of insulin resistance. Absence or inhibition of caspase-1 improves insulin sensitivity and restores normal adipocyte function. These data suggest that the inflammasome-mediated caspase-1 is an important regulator of adipocyte function and insulin sensitivity.
5. Hyperglycemia triggers activation of NLRP3 inflammasome-mediated caspase-1 and induces TXNIP-dependant IL-1β transcription in adipocytes. Accordingly, TXNIP links hyperglycemia to increased IL-1β production by the adipose tissue.
6. The inflammasome members NLRP3, ASC and caspase-1 are more abundantly present in human VAT as compared to SAT. Increased caspase-1 activity leads to elevated IL-β and IL-18 secretion levels and regulates the release of IL-6 and IL-8 by VAT. Furthermore, caspase-1 activation strongly correlates with the number of $CD8^+$ T-Lymphocytes present in adipose tissue. These data unravel caspase-1 as a novel and specific inflammatory mediator that may partly determine the pro-inflammatory character of human VAT.

Chapter 10

Nederlandse samenvatting en conclusies

Samenvatting

Obesitas wordt gekenmerkt door een overmatige ophoping van het vetweefsel, veroorzaakt door een inactieve levenstijl, toename van de voedelinname en genetische factoren die de aanleg van overgewicht bevorderen. Obesitas is geassocieerd met een verhoogd risico op het ontwikkelen van metabole aandoeningen zoals insulineresistentie, diabetes mellitus type 2 (T2DM) en hart- en vaatziekten (HVZ). Afwijkingen in de endocriene functie van het vetweefsel spelen een belangrijke rol bij het ontstaan van deze metabole aandoeningen. Zo kan een verstoorde secretie van diverse adipokines leiden tot veranderingen in de energiehuishouding en in het lipide- en glucosemetabolisme. Deze schadelijke metabole veranderingen gaan gepaard met een metamorfose van de vetweefselmorfologie, waaronder een toename van het aantal grote disfunctionele adipocyten.
Uit onderzoek is gebleken dat inflammatie een van de belangrijkste schakels is tussen obesitas en het ontwikkelen van metabole aandoeningen. Insulineresistente individuën hebben verhoogde spiegels van circulerende cytokines die sterk gecorreleerd zijn met een toename van het vetweefsel. Met name een toename van het viscerale vetweefsel draagt bij aan de verhoogde inflammatoire status in obese individuën, terwijl een uitbreiding van het subcutane vetweefsel niet schadelijk lijkt.
Pas in de begin jaren 90 van de vorige eeuw is de algehele opvatting over vetweefsel, als zijnde een orgaan dat uitsluitend betrokken is bij de opslag van overmatige hoeveelheden energie, drastisch veranderd. Verschillende studies brachten in deze periode aan het licht dat het vetweefsel van obese individuën een belangrijke bron is van pro-inflammatoire mediatoren zoals TNF-α, MCP-1, IL-6, IL-8, IL-1β en IL-18. Deze lokale ontsteking van het vetweefsel gaat gepaard met de infiltratie van verschillende soorten immuuncellen zoals macrofagen, neutrofielen en T-cell lymfocyten. Een toename van het viscerale vetweefsel vertoond meer van deze pro-inflammatoire eigenschappen vergeleken met het subcutane vetweefsel. De exacte mechanismen op welke manier obesitas bijdraagt aan het ontstaan van metabole verstoringen, middels aanhoudende inflammatie van het vetweefsel, zijn echter nog steeds onbekend.

Pathogene rol van het vetweefsel in metabole aandoeningen

Verstoorde vetweefselfunctie: afwijkende adiponectinesecretie
We hebben aangetoond dat de totale plasmaspiegels van de anti-inflammatoire en insuline-gevoeligheidseiwit adiponectine verlaagd zijn in patiënten met familiaire gecombineerde hyperlipidemie (FCH). Deze personen worden gekenmerkt door verhoogde spiegels van cholesterol, triglyceriden, apolipoproteine B en insulineresistentie. Adiponectine circuleert in diverse multimere isovormen: laag-moleculair gewicht (LMW), middel-moleculair gewicht (MMW) en hoog-moleculair gewicht

(HMW). Aanvullende studies hebben aangetoond dat de gunstige metabole en anti-inflammatoire effecten van adiponectine voornamelijk gereguleerd worden door de HMW-isovorm van adiponectine. Vandaar dat een beschermende adiponectineprofiel in de circulatie vaak wordt uitgedrukt als de verhouding tussen HMW en de totale adiponectinespiegel, terwijl een ongunstige adiponectinespiegel wordt uitgedrukt als de verhouding tussen de LMW- en HMW-isovorm van adiponectine. Wij hebben onderzocht of een verlaagd plasmaniveau van de totale adiponectinespiegel in personen met FCH gepaard ging met een verandering van de adiponectine-multimeerdistributie. Daarnaast werd de associatie bestudeerd tussen de plasmaconcentraties van de verschillende adiponectine-isovormmultimeren en de aanwezigheid van HVZ bij patiënten met FCH (**hoofdstuk 2**).
De verminderde plasmaniveaus van de totale adiponectinegehalte in patiënten met FCH ten opzichte van gezonde controles waren voornamelijk toe te wijzen aan een afname van zowel de LMW- als de HMW-isovormen van adiponectine. Echter, de verhouding tussen de HMW-isovorm en de totale adiponectine en de LMW/HMW adiponectine-verhouding verschilden niet tussen FCH en controle proefpersonen. De verdeling van de adiponectine-isovormen verschilde significant tussen mannelijke en vrouwelijke proefpersonen in beide studie-populaties, waar een gunstigere verdeling werd waargenomen bij de vrouwen. Een interessante observatie was dat binnen de groep vrouwen met de diagnose FCH, de aanwezigheid van HVZ gecorreleerd was met een vermindering van de HMW-isovorm van adiponectine in vergelijking met vrouwen zonder HVZ (HMW/totaal adiponectin: 34.2 ± 10% vs. 46.0 ± 7.1% voor respectievelijk FCH-vrouwen met en zonder HVZ; LMW/HMW adiponectine-verhouding: 1.3 ± 0.8 en 0.9 ± 0.7 voor respectievelijk FCH-vrouwen met en zonder HVZ). Vanwege de anti-inflammatoire eigenschappen die zijn toegeschreven aan adiponectine zoals de vermindering van de NF-κB activiteit, kan een nadelige adiponectine-isovorm secretieprofiel vanuit het vetweefsel een pro-inflammatoire omgeving bevorderen die het risico op het ontwikkelen van HVZ vergroot.

Vetcelhypertrofie in relatie tot vetceldisfunctie
Obesitas is geassocieerd met hypertrofische vetcellen die gekenmerkt worden door pro-inflammatoire eigenschappen. Dit bevordert de infiltratie van macrofagen in het vetweefsel wat uiteindelijk kan leiden tot de ontwikkeling van insulineresistentie. De thiazolidinediones (TZDs) vormen een klasse van farmacologische verbindingen die gebruikt worden voor de behandeling van T2DM. Deze behandeling heeft direct invloed op het vetweefsel door verbetering van de vetceldifferentiatie en glucose-homeostase veroorzaakt door PPARγ-activatie. In een studie met congenitale bijnierhyperplasie (CAH) patiënten die leiden aan insulineresistentie, onderzochten we de effecten van de TZD pioglitazone op insulinegevoeligheid, subcutane vetceloppervlak en vetweefsel-specifieke genexpressie profielen (**hoofdstuk 3**). Voorafgaand aan de behandeling vertoonde de gemiddelde subcutane vetceloppervlak duidelijke

inter-individuele verschillen en een postitieve correlatie met het percentage rompvet (r=0.73; *P*-waarde=0.01), plasmaleptinegehalte (r=0.70; *P*-waarde=0.01) en MCP-1 genexpressieniveaus in het vetweefsel (r=0.57; *P*-waarde=0.05). Verder was de vetceloppervlak negatief gecorreleerd met de systemische insulinegevoeligheid (r=-0.60; *P*-waarde=0.04) en met de genexpressie van de glucose-opname transporter (GLUT)-4 in het vetweefsel (r= -0.65; *P*-waarde=0.03). Behandeling met pioglitazone heeft geresulteerd in een verbetering van de systemische insulinegevoeligheid wat gepaard ging met een toename van de BMI (met 7 ± 1.1 kg/m^2; *P*-waarde=0.06) en de subcutane vetceloppervlak (met 497 ± 625 μm^2; *P*-waarde<0.05). Bovendien waren de genexpressie niveaus van de insulinegevoeligheids markers adiponectine en GLUT-4 toegenomen, terwijl de expressie van het pro-inflammatoire gen MCP-1 was verlaagd. Interessant is dat het percentage rompvet, plasmaleptinespiegels en MCP-1 genexpressie niet langer postief waren gecorreleerd met de subcutane vetceloppervlak. Dit suggereert dat de verslechterde metabole kenmerken van de hypertrofische vetcellen verminderd zijn na behandeling met pioglitazone, ondanks een toename van de gemiddelde vetceloppervlak. Wij veronderstellen dat de vergroting van de vetceloppervlak onder invloed van pioglitazone veroorzaakt wordt door een herverdeling van het vet vanuit het viscerale naar het subcutane opslagdepot. Deze herverdeling kan het pro-inflammatoire karakter van het viscerale vet verminderen door een afname van het aantal grote vetcellen en een toename van het percentage kleine insulinegevoelige vetcellen in dit specifieke vetdepot.

Inflammasoom-gemedieerde caspase-1 activatie in het vetweefsel

IL-18 beschermt tegen de ontwikkeling van insulineresistentie veroorzaakt door obesitas

Inflammatie en insulineresistentie veroorzaakt door obesitas wordt gekenmerkt door verhoogde cytokinespiegels in de circulatie. Het wordt algemeen aangenomen dat deze, door obesitas geïnduceerde, ontstekingsmediatoren afkomstig zijn uit de toegenomen hoeveelheid vetweefsel. Echter, tijdens obesitas kan het vet zich ook ophopen in andere weefsels zoals de lever waar het ook een onstekingsreactie kan veroorzaken.

Cytokinen kunnen tegengestelde effecten hebben op de insulinegevoeligheid. Waar TNF-α, IL-6, IL-8 en IL-1β insulineresistentie bevorderen, daar lijken andere cytokinen dit proces te remmen. Plasmaspiegels van de belangrijke IL-1-familieleden IL-1β en IL-18, zijn verhoogd tijdens obesitas en geassocieerd met insulineresistentie. Van IL-18 is echter aangetoond dat het de insulinegevoeligheid verbetert en de ontwikkeling van obesitas tegen gaat mede door het beheersen van de voedselinname, maar de onderliggende moleculaire mechanismen hiervan zijn nog onbekend. Daarom is

besloten om de bijdrage van IL-18 aan de door obesitas veroorzaakte inflammatie en insulineresistentie te bepalen, middels het voeren van een hoog-vetdieet aan zowel wild-type als IL-18-deficiënte dieren. (**hoofdstuk 4**). Tot onze verbazing was de gewichtstoename na het voeren van een hoog-vetdieet gelijk in zowel de IL-18-/- en de wild-type dieren. Wel waren deze IL-18-deficiënte muizen meer insulineresistent vergeleken met de wild-type muizen. Bovendien vertoonden de IL-18-deficiënte muizen na het eten van een hoog-vetdieet meer metabole afwijkingen zoals hogere spiegels van VLDL-triglyceriden in de circulatie en lagere plasmaniveaus van adiponectine, vergeleken met de wild-type controle muizen. Aangezien IL-18 hoog tot expressie kwam in zowel het vetweefsel als in de lever, was er het vermoeden dat deze weefsels een belangrijke rol konden spelen in de IL-18-afhankelijke effecten op de insulinegevoeligheid. Een interessante observatie was dat als de IL-18-/- muizen werden behandeld met het recombinant IL-18-eiwit, dit resulteerde in een verbetering van de hepatische insulinegevoeligheid. Een gedetailleerde analyse van de lever-specifieke effecten van IL-18 tijdens de ontwikkeling van hoog-vet geïnduceerde obesitas, leidde niet tot een verschil in de mate van leversteatose tussen beide genotypes. Echter, de plasma ALT-waarden waren duidelijk verhoogd in deze IL-18-/- muizen vergeleken met de wild-type muizen, wat wijst op leverschade. Ook de vetweefselmorfologie was niet verschillend tussen beide muismodellen. Verder was er sprake van een verrassende afname van de macrofaaginfiltratie en MCP-1-waarden in het vetweefsel van de IL-18-/- muizen gevoed met een hoog-vetdieet. Toch waren de genexpressieniveaus van het pro-inflammatoire cytokine IL-6 verhoogd en van de anti-inflammatoire cytokine IL-1Ra verlaagd in het vetweefsel van deze dieren. Alles bij elkaar suggereren deze bevindingen dat IL-18 de insulinegevoeligheid reguleert, onafhankelijk van leversteatose en macrofaaginfiltratie in het vetweefsel. IL-18 lijkt direct de hepatische insulinegevoeligheid te verbeteren en een tekort aan IL-18 gaat gepaard met een lagere adiponectinesecretie dat insulineresistentie kan bevorderen.

Inflammasoom/caspase-1 reguleren insulinegevoeligheid in het vetweefsel
De voorlopers van IL-1β en IL-18 worden verwerkt tot hun actieve vorm door een cysteïne protease genaamd caspase-1. Caspase-1 raakt zelf geactiveerd als het wordt gesplitst door een intracellulair eiwitcomplex dat het inflammasoom wordt genoemd. Dit complex bestaat uit een (NOD)-like-receptor (NLR) familielid NLRP3 en een eiwit genaamd ASC. Aangezien IL-1β en IL-18 beide invloed hebben op de metabole homeostase en vanwege de essentiële rol van inflammasoom-gemedieerde caspase-1 bij de activatie van deze twee cytokinen, bepaalden wij de rol van caspase-1 in de vetweefselfunctie (**hoofdstuk 5**). Obese muismodellen met insulineresistentie werden gekenmerkt door een verhoogde activatie van caspase-1 in het vetweefsel samen met toegenomen niveaus van IL-1β en IL-18 in het vetweefsel. Een vermindering van caspase-1 in vetcellen resulteerde in een verbetering van de adipogenese (verhoogde genexpressie van PPAR-γ, adiponectine en GLUT-4) en de insulinegevoe-

ligheid, bepaald door een verhoging van de fosforylering van het eiwit AKT (mock vs. caspase-1 siRNA, 0.95 ± 0.14 vs. 2.10 ± 0.16; *P*-waarde<0.005). Deze effecten van caspase-1 werden waarschijnlijk veroorzaakt door de rol van dit enzyme in IL-1β-activatie. Dit werd ondersteund doordat behandeling van de vetcellen met een antilichaam tegen IL-1β dezelfde effecten op insulinegevoeligheids teweeg brachten als die werden gezien ten gevolge van caspase-1-remming. Aan de andere kant werd er geen effect op de adipogenese waargenomen na behandeling van de vetcellen met recombinant IL-18. De afwezigheid van NLRP3 reproduceerde dezelfde resultaten als die werden waargenomen in de caspase-1-/- dieren zoals een verbeterde vetceldifferentiatie en vetweefselinsulinegevoeligheid. Overeenkomstig met de toegenomen insulinegevoeligheid in het vetweefsel was de IL-1β-secretie vanuit het vetweefsel in caspase-1-deficiënte en NLRP3- deficiënte dieren afgenomen. *In-vivo* experimenten met caspase-1-/- muizen bracht een verbetering van de systemische insulinegevoeligheid aan het licht vergeleken met wild-type muizen. Gelijksoortige bevindingen werden gedaan in Ob/Ob muizen, die gekenmerkt worden door overgewicht en insulineresistentie, na behandeling met een caspase-1-inhibitor zoals een verbeterde insulinegevoeligheid en een afname van het lichaamsgewicht. Het ontbreken van caspase-1 heeft geleid tot een vermindering van de vetmassa en kleinere adipocyten (gemiddelde vetceloppervlak van wild-type muizen 966.8 μM^2, caspase-1-/- muizen 629.16 μM^2; *P*-waarde<0.001). Bovendien bleek uit een indirecte calorimetrische analyse dat de caspase-1-/- dieren een hogere vetverbranding vertoonden. Deze resultaten tonen aan dat de inflammasoom-gemedieerde caspase-1-activering in het vetweefsel tijdens obesitas leidt tot een verhoogde IL-1β-productie en bijdraagt aan het ontstaan van vetweefsel-specifieke en systemische insulineresistentie. Aan de andere kant zorgt de afwezigheid of remming van caspase-1 voor een verbetering van de insulinegevoeligheid en vetweefselfunctie, wat zou kunnen betekenen dat dit als een therapeutisch doelwit kan dienen voor de behandeling van obesitas en T2DM.

Hyperglycemie-gemedieerde caspase-1-activatie en IL-1β-secretie in het vetweefsel

Het belang van IL-1β in, de door obesitas opgewekte, ontsteking en in de ontwikkeling van insulineresistentie in het vetweefsel zijn reeds goed gedocumenteerd in verschillende studies. Daarnaast hebben wij aangetoond dat de inflammasoom/caspase-1 de functie van het vetweefsel beïnvloedt tijdens de ontwikkeling van obesitas (**hoofdstuk 5**). Echter, de signalen die leiden tot caspase-1-activering in het vetweefsel zijn nog niet bekend. Een recente studie heeft aangetoond dat hyperglycemie een interactie induceerde tussen thioredoxin-interacting protein (TXNIP) en NLRP3, wat uiteindelijk leidde tot caspase-1-activering en IL-1β-productie door beta-cellen in de pancreas van de muis. Vervolgens hebben wij gekeken of er een soortgelijk mechanisme bestaat in humane vetcellen en intact vetweefsel (**hoofdstuk 6**). Behandeling van humaan intact vetweefsel en primaire vetcellen met hoge concentraties glucose,

resulteerde in een toename van de IL-1β-transcriptieniveaus (respectievelijk 7.5×; *P*-waarde<0.01 en 1.7×; *P*-waarde<0.05), intracellulaire eiwitniveaus van pro-IL-1β (respectievelijk 2.0×; *P*-waarde<0.01 en 1.5× *P*-waarde<0.05) en de secretie van bioactief IL-1 (respectievelijk 2.0×; *P*-waarde<0.01 en 3.0×; *P*-waarde<0.05). Bovendien nam de caspase-1-activiteit toe met 10% (*P*-waarde<0.05) en waren de NLRP3-eiwitniveaus verhoogd in humane adipocyten die behandeld werden met hoge concentraties glucose. Naast caspase-1 en NLRP3, waren ook de TXNIP-eiwitniveaus gestegen in zowel humaan intact vetweefsel als in vetcellen als reactie op het hoge glucosegehalte (respectievelijk 2×; *P*-waarde<0.05 en 5×; *P*-waarde<0.01). De verhoogde secretie van bioactief IL-1 in vetcellen geïnduceerd door een hoog glucosegehalte, verminderde wanneer deze cellen behandeld werden met siRNA tegen TXNIP. Dit ging gepaard met een significante 2-voudige afname in IL-1β-transcriptieniveaus en intracellulaire niveaus van pro-IL-1β. Echter, de uitschakeling van TXNIP had geen effect op de caspase-1-activering wanneer de vetcellen gestimuleerd werden met hoge concentraties glucose.
Deze resultaten tonen aan dat hyperglycemie zou kunnen dienen als een signaal voor NLRP3-inflammasoom-gemedieerde caspase-1-activering. Bovendien zorgen de hoge concentraties glucose voor de inductie van vetcel-specifieke TXNIP-expressie, dat vervolgens leidt tot een verhoging van de IL-1β-transcriptie. TXNIP lijkt echter niet direct caspase-1-activering te stimuleren in het vetweefsel. Concluderend, hyperglycemie induceert zelfstandig zowel TXNIP-gereguleerde IL-1β-transcriptie als-caspase-1-activering, die samen leiden tot een verhoogde productie van IL-1β dat insulineresistentie kan bevorderen.

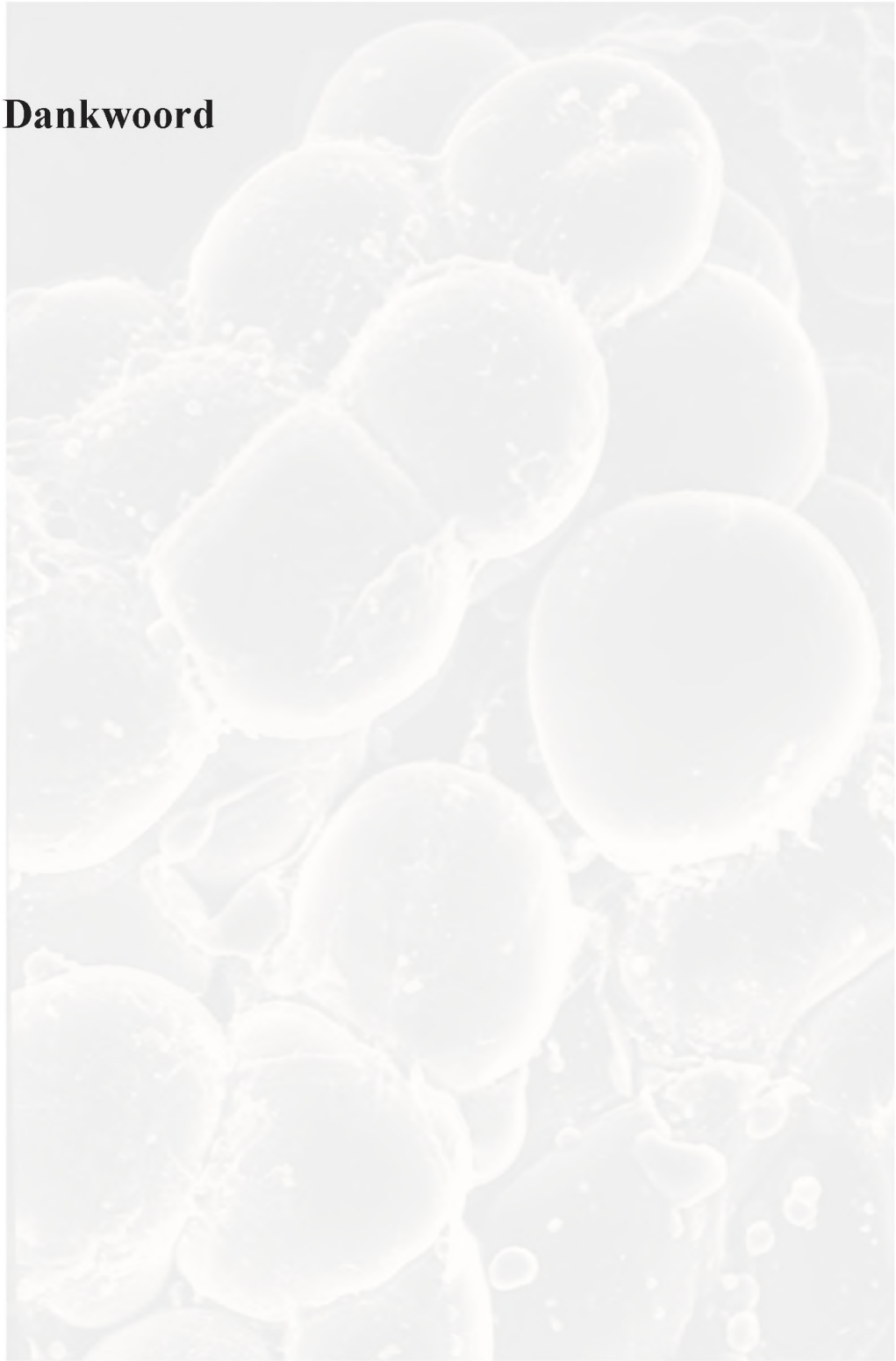
Inflammasoom en caspase-1-activering in het viscerale vetdepot
Er wordt beweerd dat het viscerale vetweefsel meer bijdraagt aan de verhoogde pro-inflammatoire cytokinen in de circulatie van obese personen dan subcutaan vetweefsel. Deze pro-inflammatoire eigenschappen van het viscerale vetweefsel kunnen vooral worden toegeschreven aan de toegenomen infiltratie van verschillende ontstekingscellen die de productie van diverse ontstekingmediatoren bevorderen. Hoewel de door het inflammasoom-gereguleerde IL-1β en IL-18 ook in verband worden gebracht met obesitas en insulineresistentie, en ze deels afkomstig zijn uit het vetweefsel, is het momenteel niet bekend of de inflammasoom/caspase-1-activiteit bijdraagt aan de pro-inflammatoire status van het viscerale vetweefsel ten opzichte van het subcutane vetweefsel. Daarom hebben wij het expressieprofiel van de NLRP3-inflammasoom-componenten te bestudeerd, door middel van mRNA- en eiwitanalyses in gepaarde viscerale en subcutane vetweefselbiopten afkomstig van personen met overgewicht (**hoofdstuk 7**). In deze studie werd de cellulaire samenstelling van de stromale vasculaire fractie in het viscerale vetweefsel gekenmerkt door een toename van het aantal $CD8^+$ T-lymfocyten vergeleken met het subcutane vetweefsel (30.4% vs. 41.6%; *P*-waarde<0.05). Verder werden er geen verschillen waargenomen in het aantal

macrofagen en vetceloppervlak tussen beide vetdepots. De *ex-vivo* vetweefselkweken toonden echter aan dat de toegenomen intrinsieke inflammatoire eigenschappen van het viscerale vetweefsel geïllustreerd werden door een versterkte afgifte van IL-6, IL-8, IL-1Ra (respectievelijk 3×; *P*-waarde<0.05, 4×; *P*-waarde<0.05, en 2×; *P*-waarde<0.05) en een afname in adiponectinesecretieniveaus. Ook van belang is dat de totale secretie van IL-1β (pro-IL-1β + actieve vorm van IL-1β, 10×; *P*-waarde<0.05), bioactief IL-1 (10×; *P*-waarde<0.05) en IL-18 (3×; *P*-waarde<0.05) hoger was in het viscerale vetweefsel vergeleken met het subcutane vetweefsel.
Overeenkomstig de verhoogde secretieniveaus van IL-1β en IL-18, liet het viscerale vetweefsel een 3-voudige (*P*-waarde<0.05) toename zien in caspase-1-activiteit en een verhoogd eiwitgehalte van de inflammasoom-componenten NLRP3 (2×; ns) en ASC (2×; *P*-waarde<0.05) vergeleken met het subcutane vetweefsel. De caspase-1-afhankelijke cytokineproductie door humaan vetweefsel werd verder ondersteund door de behandeling van stukjes visceraal vetweefsel met de specifieke caspase-1-remmer pralnacasan. Dit leidde tot een verminderde afgifte van IL-1β en IL-18, maar ook van de productie van IL-6 en IL-8. De TNFα-secretieniveaus werden echter niet beïnvloed door het remmen van caspase-1. Ten slotte werd een significante positieve correlatie waargenomen tussen de caspase-1-activiteitniveaus en het percentage $CD8^+$ T-lymfocyten aanwezig in het vetweefsel (r=0.77; *P*-waarde<0.01). Deze resultaten suggereren dat caspase-1 een belangrijke bijdrage levert aan het intrinsieke pro-inflammatoire karakter van het viscerale vetweefsel via de verhoogde productie van IL-1β en IL-18, maar ook door het reguleren van de productie van IL-6 en IL-8. Bovendien is caspase-1 positief gecorreleerd met het aantal $CD8^+$ T-lymfocyten in het vetweefsel, waarvan bekend is dat ze bijdragen aan de ontsteking in het vetweefsel. Het reguleren van de infiltratie van $CD8^+$ T-lymfocyten kan een extra route zijn waarlangs caspase-1 de vetweefselontsteking beïnvloedt.

Conclusies

1. Verlaagde totale adiponectinespiegels in de circulatie van FCH-patiënten zijn niet geassocieerd met een verstoorde adiponectine-multimeer-profiel. Echter, bij vrouwen met de diagnose FCH gaat de aanwezigheid van HVZ gepaard met een meer atherogene adiponectine-multimeer-secretieprofiel vanuit het vetweefsel.
2. Een toename van de subcutane vetceloppervlak is geassocieerd met inflammatie en insulineresistentie. Deze associatie is verdwenen na pioglitazone-behandeling wat geïllustreerd wordt door een vergroting van de subcutane vetceloppervlak samen met een vermindering van de inflammatie en een verbetering van de systemische insulinegevoeligheid.
3. Het IL-1-familielid IL-18 reguleert insulinegevoeligheid zonder de ontwikkeling van obesitas te beïnvloeden en zonder dat het een postief effect heeft op de mate van lever-steatose en macrofaaginfiltratie in het vetweefsel tijdens een hoog-vetdieet.
4. Een toegenomen inflammasoom-gemedieerde caspase-1-activering in het vetweefsel tijdens obesitas leidt tot een verhoogde IL-1β-productie en draagt bij aan het ontstaan van insulineresistentie. De afwezigheid of remming van caspase-1 verbetert de insulinegevoeligheid en herstelt de normale vetcel functie. Deze gegevens suggeren dat de inflammasoom-gemedieerde caspase-1 een belangrijke regulator is van de vetcelfunctie en insulinegevoeligheid.
5. Hyperglycemie brengt een activatie teweeg van de NLRP3 inflammasoom-gemedieerde caspase-1 en induceert een TXNIP-afhankelijke IL-1β-transcriptie in de vetcellen. Op deze manier koppelt TXNIP hyperglycemie aan een verhoogde IL-1β-productie door het vetweefsel.
6. De inflammasoom-componenten NLRP3, ASC and caspase-1 komen meer tot expressie in humaan visceraal vetweefsel vergeleken met subcutaan vetweefsel. Een toename in caspase-1-activiteit leidt tot verhoogde IL-β and IL-18 secretieniveaus en reguleert de afgifte van IL-6 en IL-8 in het viscerale vetweefsel. Bovendien is caspase-1-activering sterk gecorreleerd aan het aantal $CD8^+$ T-lymfocyten die aanwezig zijn in het vetweefsel. Deze gegevens ontrafelen caspase-1 als een nieuwe en specifieke ontstekings-mediator die deels het pro-inflammatoire karakter bepaalt van het humane viscerale vetweefsel.

Dankwoord



Het klinkt heel erg cliché, maar het doen van promotieonderzoek gaat met pieken en dalen. Gedurende deze vier jaar mag ik wel zeggen dat dit ook op mij van toepassing was. Maar de dalen die je in het ondezoek tegenkomt, hebben ook voordelen. Zo heb ik gemerkt dat je er eigenlijk altijd sterker uitkomt en deze ervaring neem je weer mee in het verdere verloop van je onderzoek. Een ander voordeel is dat wanneer je een piek bereikt in je onderzoek, je hier extra van geniet. Deze zogenaamde pieken uiten zich in dingen als een mooie paper, het houden van een goede presentatie, het vinden van leuke resultaten, of gewoon een simpel schouderklopje die je krijgt voor het harde werken. Het behalen van deze pieken daar doe je het allemaal voor en dit kan je uiteraard nooit alleen bereiken. Zoals met de meeste dingen in het leven bereik je samen altijd veel meer dan in je eentje. Zo'n team van mensen op wie je altijd even terug kan vallen, die je raad geven en die je net het duwtje in de juiste richting geven, zijn echt goud waard. Dit kunnen mensen zijn in het laboratorium die je praktisch ondersteunen, leidinggevenden die je inhoudelijk de weg wijzen, maar ook de mensen van het thuisfront die altijd achter je staan. Gelukkig heb ik tijdens mijn onderzoek ook te maken gehad met zo'n gouden team, zonder wie dit proefschrift nooit tot stand zou zijn gekomen. Enkele personen wil ik graag in het bijzonder bedanken.

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Curriculum Vitae

Tim Bernardus Koenen werd geboren op 24 september 1982 te zevenaar. Na het behalen van het VWO diploma aan het Candea College in Duiven begon hij in 2001 met de studie Biomedische Wetenschappen aan de Radboud Univeristeit Nijmegen met als hoofdvak Pathobiologie. Tijdens zijn studie heeft hij onderzoekservaring opgedaan bij de afdeling Orthodontie en craniofaciale Biologie (onder begeleiding van Dr. Hans Von den Hoff), bij de afdeling nierziekten (onder begeleiding van Dr. Mabel van den Hoven en Dr. Johan van der Vlag) en bij de afdeling Research and Development van Organon (onder begeleiding van Dr. Han Gerrits). In November 2006 heeft hij zijn studie met goed gevolg afgerond en behaalde hij zijn Master of Science graad. Van januari 2007 tot en met januari 2011 was hij verbonden als junior onderzoeker aan de afdeling Algemeen Interne Geneeskunde van de Radboud Universiteit Nijmegen Medisch Centrum, alwaar dit proefschrift tot stand is gekomen. In de maanden januari tot en met april van 2011 heeft hij in samenwerking met de afdeling Chirurgie van het Canisius Wilhelimina Ziekenhuis een vetweefsel bio-bank tot stand gebracht die in de toekomst gebruikt kan worden voor het onderzoek. Na deze mooi ervaring binnen de academie, wil hij zijn carrière vervolgen in de farmaceutische industrie.

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